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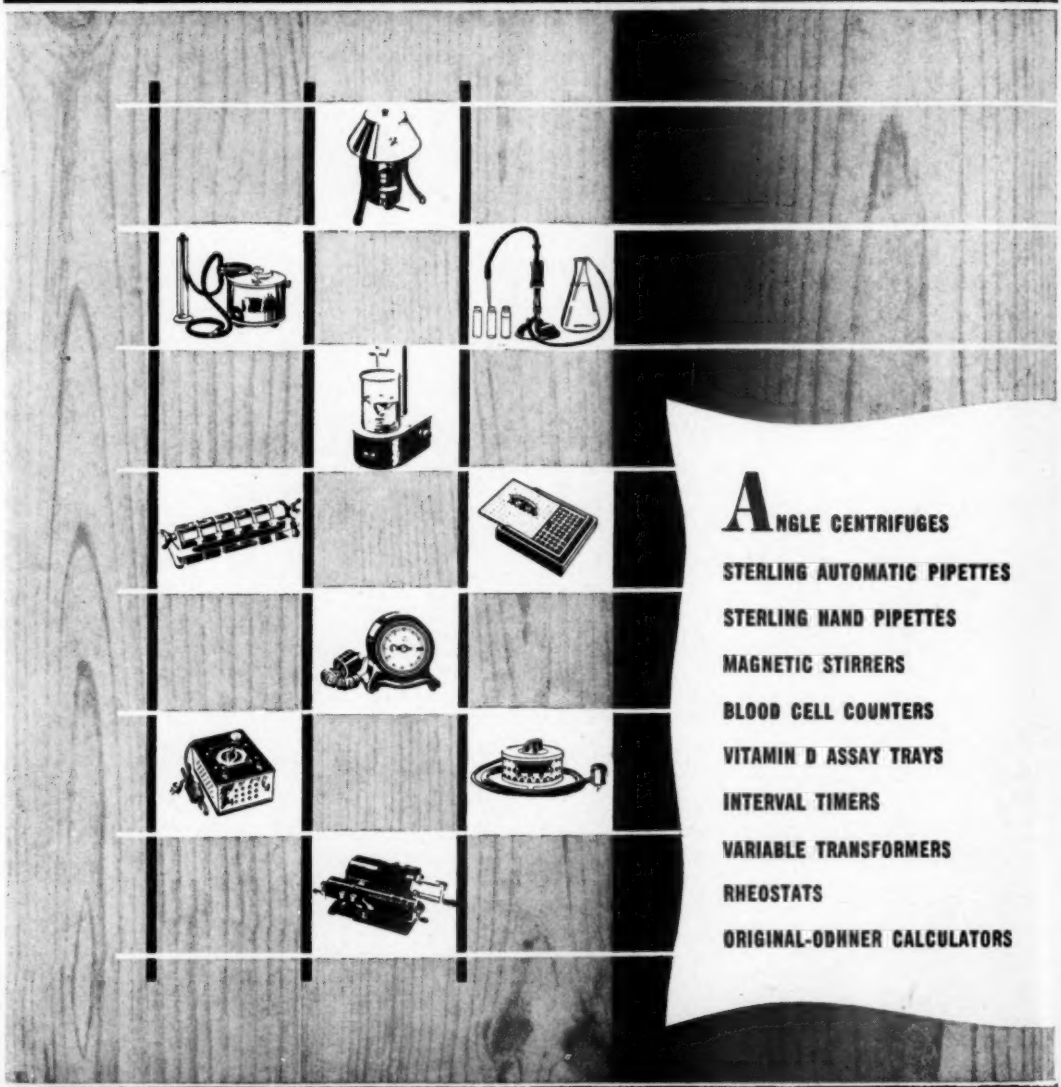

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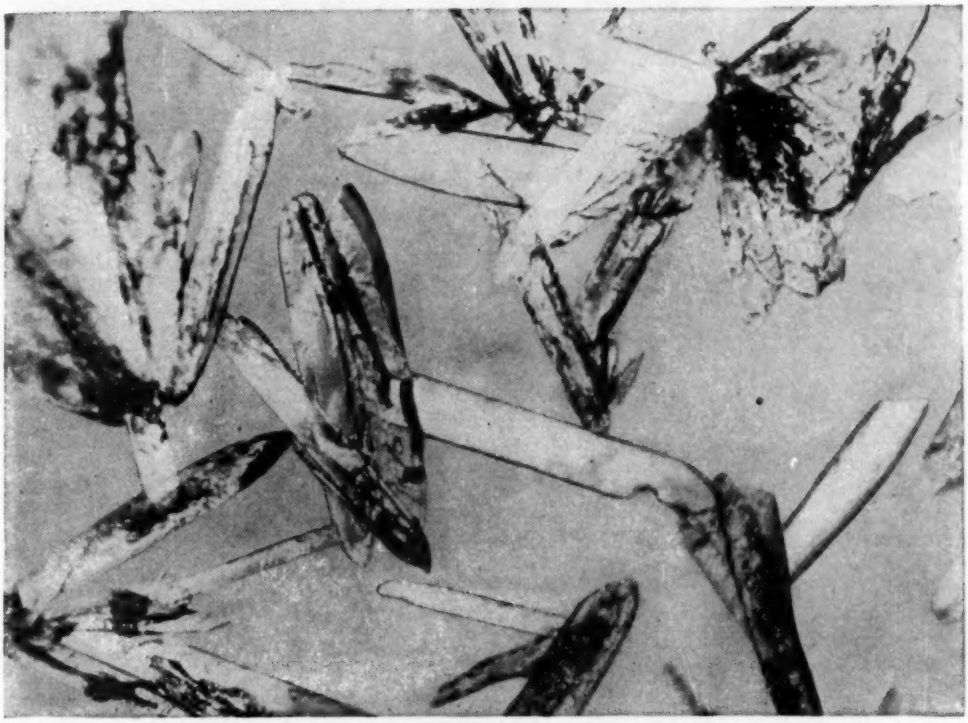

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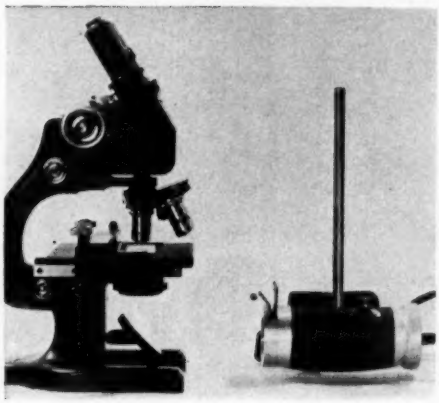

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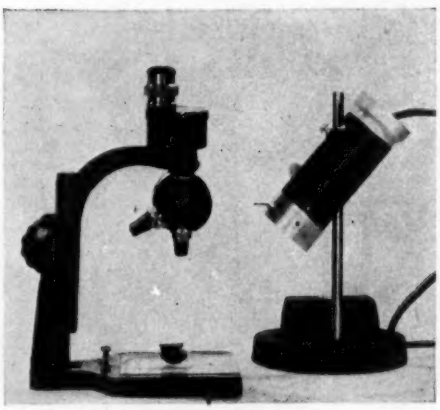

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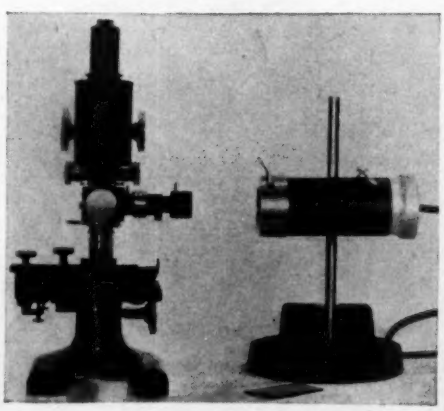

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Morphology of Bovine Spermatozoa

C. K. RAO, B.V.Sc. and G. H. HART, V.M.D., M.D.

Davis, California

CAREFUL study of bovine sperm reveals variations in morphology which may have a bearing on longevity and fertilizing ability. Review of the literature on this subject shows a great variety of techniques, stains, and classifications. From this it is difficult to differentiate between possible artifacts and normal structure changes taking place in the sperm from their origin in the seminiferous tubules to the ejaculated cells mixed with secretions of the accessory glands. Species variations are considerable, and we have confined our data to the bovine species.

Extensive accumulations of data on this species have been made possible through the widespread use of artificial insemination during recent years. Morphology is a criterion for evaluating semen when combined with sperm concentration and motility.

TECHNIQUES

This work was done with bulls in an artificial insemination unit; also reproductive organs of bulls were obtained from a nearby packing plant and examined immediately or after storage at 45 F.

One of the most important considerations in the examination of sperm is the devising of delicate techniques which limit artifacts to a minimum. In ejaculated semen with high concentration of sperm, the importance of having these cells uniformly and sufficiently dispersed over the microscopic field for the study of morphology is evident.

A factor of great importance is, thus, the technique of making semen films, and this cannot be too greatly stressed. It is essential to have clean and smooth slides with straight edges. The neck is the weakest spot in the sperm and, unless very light pressure is used, the edge of the glass slide may sever some heads. Salisbury, Willett, and Seligman[1] found that the most satisfactory method of making semen films is that in which a drop of diluted semen is spread over a clean slide by simply placing another clean slide face down on it and pulling the two apart lengthwise. This method may be of considerable value in making films of diluted semen. In cases where thick stains like opal blue (Breslau) or opal blue eosin mixture prepared according to Lasley, Easley, and McKenzie[2] are used to stain sperm by actually mixing with semen, it has been found that the films obtained by this technique[1] are too thick and lack uniformity of sperm dispersal. In making films of stained semen, we dip the straight end edge of a clean glass slide in the semen and stain mixture until enough material adheres. This edge with the slide slanted at a slight angle is then placed towards one end of the upper surface of a second slide held firmly on the table. A thin film spreads as the upper slide is pushed slowly over the lower slide. It is important to move the upper slide with uniform pressure and speed to obtain a film of uniform thickness, with an equal distribution of sperm throughout. The pressure remaining constant, the thickness of the film can be controlled by the speed with which the upper slide is pushed forward.

Mercier and Salisbury[3] investigated the influence of various fixatives and clearing agents on the proportion of abnormal sperm and found that clearing of the preparations with 1 per cent chlorazene was responsible for producing most tailless heads. Throughout our work on sperm morphology, no fixatives or clearing agents were used. Wet preparations of semen, under a coverslip, diluted with 3 per cent saline to kill the sperm (Anderson[4]) have been used to a considerable extent and found particularly valuable. Such wet films tend to reduce artifacts to a minimum. Examination of these wet films against a colored background obtained by applying 1 per cent eosin, methylene blue, or other stains on the reverse side of the slide and allowing to dry, or by inserting a light filter in the microscope,

Graduate, Madras Veterinary College, Madras, India, and graduate student, University of California (Rao); head of the Division of Animal Husbandry, College of Agriculture, University of California (Hart).

From the Division of Animal Husbandry, College of Agriculture, University of California, Davis.

affords additional clarity. When prolonged examination is necessary, drying of the film of semen can be prevented by sealing all around the coverslip with a thin layer of cedarwood oil. In such preparations, only a sufficient amount of diluted semen should be taken, so that when the coverslip is placed on the drop, and the slide slanted, the coverslip does not change its position. If thick films are made, there will be more than one layer of sperm. Excess of fluid can be removed from under the coverslip by means of blotting paper. A further refinement of this method, where it is desired to examine stained sperm, consists in placing a drop of some easily diffusible stain like eosin or hematoxylin at the edge of one point of the coverslip and sealing all around with cedarwood oil. In such preparations, the uniform dispersal of sperm may be disturbed by the current set up in diffusion of the stain, but there are enough sperm, either single or in small groups, for observation. Such preparations also give an insight into the affinity of different parts of the sperm for various stains and render unnecessary other detailed and laborious techniques.

Another type of wet preparation is made by mixing a drop of diluted semen and stain with a toothpick. After one minute, sufficient material from this semen-stain mixture is transferred to a drop of saline on another slide which dilutes the stain and facilitates observation. Opal blue alone is the most useful stain to bring out the protoplasmic cap and the protoplasmic drop. A drop of semen is mixed with a drop of opal blue and the mixture allowed to stand for one minute before dry films are made, as described earlier. It has not been found necessary to filter or boil the stain before using. Certain other stains, like opal blue eosin mixture or eosin alone, may be used with this technique in staining the drop. When opal blue is used, the protoplasmic drop shows itself as either a white, unstained area or as a pale blue mass. If the mixture is allowed to stand for ten minutes, or the sperm stained by the diffusion technique, all protoplasmic drops take the stain. Eosin is useful with fresh epididymal sperm in showing that the protoplasmic drop is not a bulging of the connecting piece, but rather an extracellular mass with the connecting piece continuing right through it.

In opal blue dry films the anterior half of the sperm head stains pale blue or remains unstained, while the posterior half stains deep blue.

Using the diffusion technique with a drop of opal blue touched to the wet preparation, the staining reaction of the spermhead can be watched as the stain mixes with the semen. The sperm start taking the stain first at the base of the head, and the stained area rapidly extends up to the middle of the head, where it is limited by a sharp and clear-cut straight line. When the posterior half of the sperm head is observed under oil immersion, it can be seen that the deepest stain is taken by the anterior three fourths, while the remaining one fourth is lightly stained. After several hours, the anterior half of the head finally approximates the posterior half in color. When eosin or safranine is used, the whole of the head quickly stains uniformly pink or orange.

Development of Sperm

Our work with testicular preparations did not go into a study of the sex cells prior to their becoming free in the lumen of the tubules. At their origin, the primordial sex cells or spermatogonia are arranged in layered fashion in the periphery of the thick-walled seminiferous tubules, and each cell has the normal number of chromosomes for the species. With maturation, well described for man by Arey,[5] they move from the periphery toward the lumen of the tubules, enlarge, and become the primary spermatocytes. These larger cells then divide into two smaller cells with reduction of the chromosome numbers to one-half the normal number for the species, and these cells are the secondary spermatocytes. They again divide producing four smaller spermatids. These spermatids lie in close association with the second type of cells in the tubules called the Sertoli or nurse cells, from which they appear to receive nutrition, and, during this association, they mature into spermatozoa and become free in the lumen of the tubules. This involves changes in the cell shape. The nucleus condenses and forms the bulk of the sperm head; the flagellum grows out from the neck. The remainder of the cytoplasm is cast off after a sheath closely encircles the flagellum as a capsule well down toward the end. It stops abruptly, and the flagellum continues as a naked ending. Thus, the small sperm cells do not resemble other body cells as do the large ovarian cells. The neck is very short, and the tail consists of three parts; a connecting piece, midpiece, or body, about the length of the head; a much longer chief piece constituting three-fourths the length of the entire cell; and the end piece, terminal filament, or naked flagellum, constituting the end of the axial filament which courses the entire length of the tail.

The factors responsible in the movement of spermatozoa from the testicles to the ampullae have been the subject of much discussion. The constant production of spermatozoa and

liquid secretions along with ciliary action within the epididymides are the important factors. Contraction of muscular fibers in the walls of the epididymides and vasa deferentia also aid their propulsion.[6]

TABLE 1—Protoplasmic Droplets on Sperm from the Tail of the Epididymis Diluted with Saline and Allowed to Stand at Room Temperature for Four Hours

		Droplets		
		Anterior	Posterior	None
Bull 1	Before dilution	12	63	25
	After dilution	9	30	61
Bull 2	Before dilution	6	61	33
	After dilution	4	19	77
Bull 3	Before dilution	3	15	82
	After dilution	3	4	93

SPECIAL CHARACTERISTICS OF BOVINE SPERM

In the bovine and other species, many sperm in the lumen of the seminiferous tubules have a rather large protoplasmic drop surrounding the connecting piece of the tail just posterior to the head (*see* fig. 1).

In testicular films, we have observed this drop surrounding the posterior half of the head in some instances. The drop has been described by many workers. In the testicle and the head of the epididymis, the drop is present at the anterior end of the connecting piece, completely surrounding it. In the tail of the epididymis, in the majority of sperm, it is located at the posterior end of the connecting piece. In the body of the epididymis, there is a varying proportion of the two types of sperm (*see* fig. 2, 3, and 5). The droplet gets smaller in size as it reaches the posterior end of the connecting piece and does not seem to completely encircle it. The ripened sperm pass along the lumen of the vas deferens, and accumulate in the ampulla where the drop is discarded from many sperm and can be seen loose in the microscopic field in some preparations (*see* fig. 4). Throughout this progression to the ampulla the sperm are practically nonmotile, but motility can be readily demonstrated by diluting the contents of the lumen of the epididymis with physiologic saline or injecting saline solution into the canal or infiltrating it into the testicular tissue. In the ampulla, motility in fresh preparations can be demonstrated, but this can be greatly increased by diluting the contents of the ampulla with physiologic saline.

The significance of the protoplasmic drop is not clear at the present time. In the bovine, its permanence is not necessary for life of the sperm, and it is usually absent from ejaculated sperm. We have observed motility in epididymal sperm lacking the drop. On the other hand, Merton[7] has shown in the mouse that sperm from the epididymis which have lost the protoplasmic drop quickly die, but its absence is not harmful to mature ejaculated sperm. In the boar, the protoplasmic drop is present in epididymal sperm, and Lasley and Bogart[8] have shown that epididymal sperm of this species survive *in vitro* for a longer period of time than do ejaculated sperm from the same male stored under identical conditions.

Redenz,[9] Lagerlöf,[10] and others were of the opinion that sperm having the drop were immature. Selivanova[11] did not view the change in position of the drop as a criterion of maturity. We observed that sperm with the drop at the posterior end of the connecting piece have normal motility and are able to cast it off. Those with the drop at the anterior end are incapable of active motility and cannot cast it off. From Lagerlöf's[12] work Anderson[4] states, "It appears that the presence of more than 2 or 3 per cent of immature sperm indicates pathological changes in the genital organs and reduced fertility." Gunn *et al.*,[13] in a study of sections from the rete testis, as well as sperm in the epididymis and in ejaculated semen of abnormal rams, confirm this and believe that droplets are an indication of abnormal spermatogenesis.

TABLE 2—Protoplasmic Droplets on Sperm in Different Locations of the Male Genital Tract

		Droplets		
		Anterior	Posterior	None
Guernsey	Head of Epididymis	12	71	17
	Tail of Epididymis	0	8	92
	Ampulla	0	1	99
Guernsey	Head of Epididymis	86	0	14
	Tail of Epididymis	3	65	32
	Ampulla	3	9	88
Hereford	Head of Epididymis	26	63	11
	Tail of Epididymis	2	28	70
	Ampulla	0	2	98
Hereford	Head of Epididymis	9	25	66
	Tail of Epididymis	0	15	85
	Ampulla	1	5	94
Hereford	Head of Epididymis	96	0	4
	Tail of Epididymis	0	56	44
	Ampulla	0	5	95
Aberdeen-Angus	Head of Epididymis	46	21	33
	Tail of Epididymis	2	54	44
	Ampulla	0	4	96

In our study of sperm maturation, it seemed logical to consider the descent of the drop to be involved with the development of the sperm. When they have reached maturity, the drop has moved to the posterior end of the connecting piece or has been cast off without particular reference to the location of the sperm. It appears to perform a nutritive

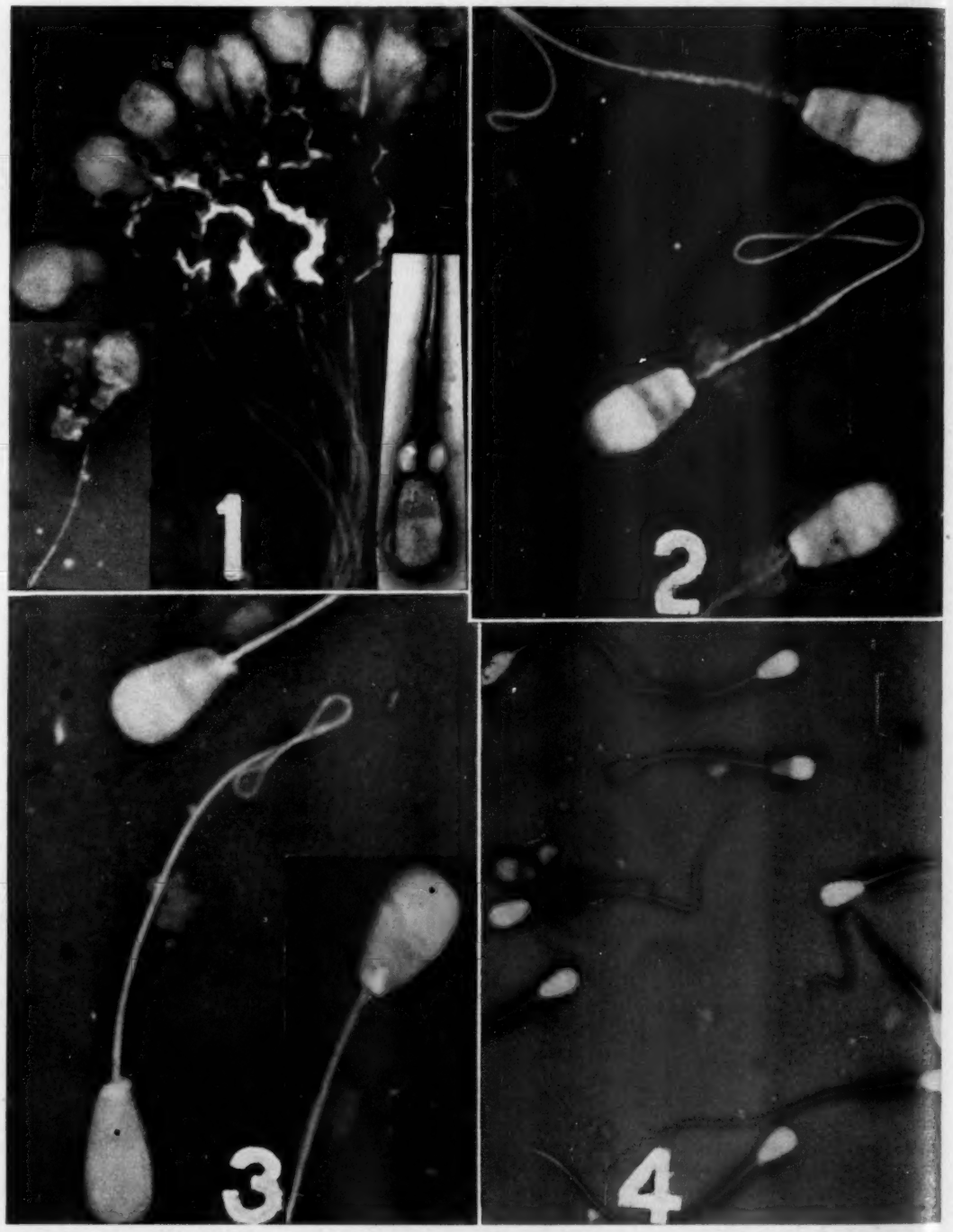


Fig. 1—Dry film (opal blue Breslau). Mass of developing sperm in lumen of seminiferous tube. Note individual sperm lower right with protoplasmic cap, droplet and tortuous connecting piece. Also sperm lower left with filiform connecting piece and extruding cytoplasmic remnant. × 1,500.

Fig. 2—Dry film (opal blue Breslau). Sperm from head of epididymis. Showing droplet still close to the head with tortuous connecting piece and distinct cap. × 2,000.

Fig. 3—Dry film (opal blue Breslau). Sperm from body of epididymis. Note droplet at different locations on connecting piece. × 2,000.

Fig. 4—Dry film (opal blue Breslau). Sperm from ampulla, many without droplets. Some loose drops in the field. × 750.

function in the development of the connecting piece. The latter is very thin or undulated when the drop is at the neck and becomes straight and thickens as the drop moves down (fig. 2 and 8). The function of the drop seems to have been completed when it reaches the posterior end of the connecting piece. The presence of droplets on a considerable percentage of ejaculated sperm might be assumed to result from defects in spermatogenesis, but we have never observed this. On the other hand, a small number, less than 1 per cent of such sperm, is normal. Active motility is all that is necessary for the sperm to rid themselves of the drop when located at the posterior end of the connecting piece. This is aided by the mechanism of ejaculation. In unstained, wet coverglass preparations of dead sperm from the tail of the epididymis we have observed posterior drops to be mechanically removed as the preparation dried; air bubbles extend over the sperm, and the coverglass is pulled closer to the slide.

One hundred sperm from the tail of the epididymis from 3 bulls were counted for drops. Material from the same epididymides was immediately diluted with saline and allowed to stand at room temperature for four hours during which time quite active motility of the cells developed. A second count was then made with results as shown in table 1.

We credit the activity of these cells under dilution to have been responsible for the marked increase in cells lacking the drops.

In order to further study the distribution of the drop in several regions of the genital passages, the reproductive organs of 6 bulls were obtained at a packing house on the same day. The animals consisted of 3 Herefords, 2 Guernseys, and 1 Aberdeen-Angus, all between 4 and 6 years of age. Opal blue stained dry smears were made from the head and tail of the epididymis and the ampulla of each animal. One hundred sperm on each slide were counted for the presence and location or the absence of drops. All the droplets that had moved from the anterior end of the connecting piece were classed as posterior drops. The data are given in table 2.

These data show individual variations, but in all cases a high percentage of sperm cells in the ampulla were devoid of drops. Also it was evident that sperm with drops attached were not confined to any particular part of the tract, although the anterior drops were reduced to a minimum in the tail of the epididymis and were practically absent in the ampulla.

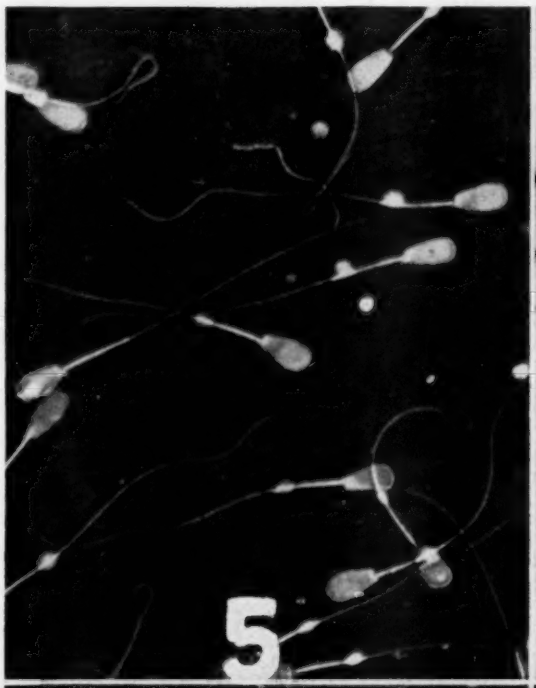
In the seminiferous tubules, we have observed the extrusion of the cytoplasmic remnant, containing the Golgi apparatus, from the tail of the sperm as described in the development of human sperm by Arey.[5] The head differentiates early, before the sperm are free in the lumen of the seminiferous tubules, into an anterior head cap or acrosome and posterior head cap or postnuclear cap, with a distinct straight dividing line between them, about the middle of the head, which can be seen even in unstained preparations.

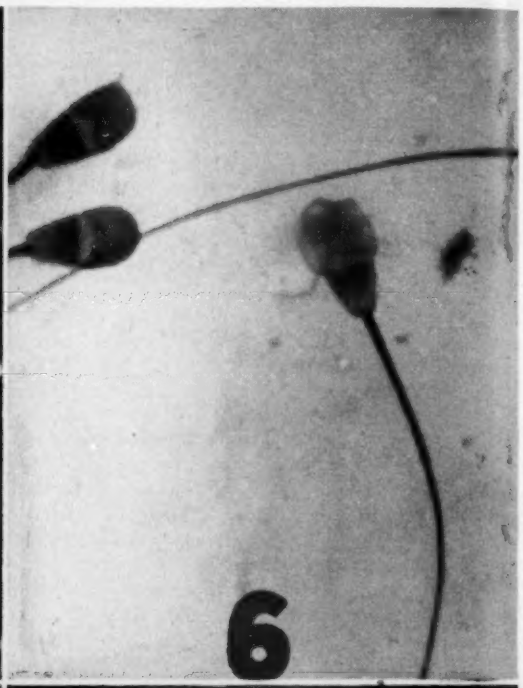
When viewed on edge, the sperm head is elongate, slightly biconcave, and about the same thickness as the connecting piece. When motility is most active it always assumes this position. When examined on flat, the head is seen to be elongate oval in shape, with the broad end facing anteriorly. In a stained preparation under high magnification, the base of the head is seen to be dished in to receive the connecting piece, which is joined by a very short neck. Occasionally the anterior and posterior halves of the head will be found broken asunder at the separating line.

In sperm stained by opal blue or opal blue eosin mixture, when examined under oil immersion, a peculiarly irregular protoplasmic material is found extending around the acrosome (fig. 2 and 6). This process has been termed the protoplasmic cap by Baylor, Nalbandor, and Clark[14] who reported its presence in ejaculated bovine sperm examined under the electron microscope. The extent of its development is highly variable, from a slight roughening at the front end of the sperm to a very pronounced and clear cap extending all round the anterior half of the head, through which the outline of the acrosome can be seen. This cap can be faintly seen even in unstained preparations. Ordinarily when stained with opal blue or opal blue eosin mixture, the cap takes a very light stain. By using the special diffusion technique, where the stain is allowed to act on the sperm for several hours, it can be demonstrated that the cap exhibits a delayed staining reaction, and is then readily seen (fig. 6). This protoplasmic cap is most prominent in the sperm from the testis and epididymis and can be demonstrated without much difficulty.

The posterior margin of the sperm head, where it joins the neck, is seen as a dark line in stained as well as unstained preparations, and corresponds to the neck granule described by Arey[5] for the human sperm.

The connecting piece of the tail under the high, dry lens is bounded on either side by a dark, prominent wall, with the filament running through the center. These dark lines noted correspond to the mitochondria, which are arranged all along the length of the connecting piece of the human sperm.[5] Posteriorly, the connecting piece is sharply limited by a





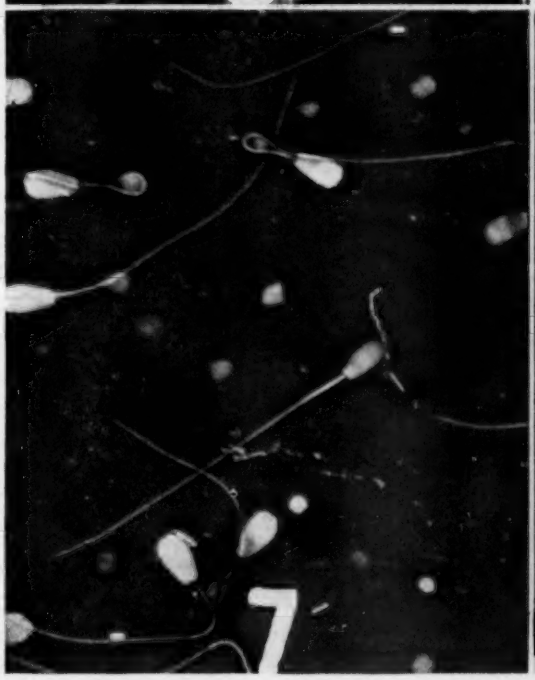


Fig. 5—Dry film (opal blue Breslau). Sperm from tail of epididymis with droplets at posterior end of connecting piece, and some free. × 750.

Fig. 6—Wet preparation. Stained by diffusion technique. (opal blue Breslau). Ejaculated sperm showing one cell with a well defined cap and outline of acrosome beneath. × 1,500.

Fig. 7—Dry film (opal blue Breslau). Smear from same sample of epididymal sperm as figure 5 after storage at 45 F. for twenty-four hours. Note partial and complete bending of tails. × 750.

Fig. 8—Dry film (opal blue Breslau). Ejaculated sperm after twenty-four hours storage at 45 F. showing bent tail in one sperm. Note the distinct outline of the connecting piece. × 1,500.

clear, dark line which represents the posterior centriole, which extends around as a ring. The anterior centrosome is found as a dark, transverse line limiting the connecting piece anteriorly. Figure 8, taken under oil immersion, shows the distinct outline of the connecting piece. In the anterior head cap, we have not been able to demonstrate the vacuole present in human sperm. We have not been able to demonstrate a lipoid capsule in bovine sperm.

Functional Specialization of Sperm.—This highly differentiated cell has its various parts specialized for specific functions. The posterior half of the head contains the nuclear elements. The anterior cap, with the irregular protoplasmic material surrounding it, in many of the sperm may be involved in the enzyme action associated with fertilization of the ovum. Greenberg and Cargill[15] showed that hyaluronidase is present in semen of rabbits and suggested that this is concerned with dispersal of corona radiata cells persisting around the expelled ovum. Leonard and Kurzrok[16] injected extracts of bull testes containing hyaluronidase into rats and produced an antiserum which inhibited this enzyme action on corona radiata cells *in vitro* under similar dilutions at which normal rat serum had no effect. The protoplasmic cap surrounding the acrosome leads us to suggest that this, along with the acrosome, may be a source of the enzyme in these cells.

The tail is chiefly concerned with locomotion. Cody[17] demonstrated that motility is independent of the head by severing the head without destroying the tail motility. We have occasionally observed motile, headless sperm in highly diluted semen, severed in the process of preparation. Injury to the tail causes loss of motility. The strong impulse for motility is not ordinarily destroyed even by serious abnormalities. Popa[18] and Marza[19] suggest that the posterior centriole in human sperm is the regulator of this function.

Abnormal Sperm.—A great amount of data have been published regarding the importance of abnormal sperm in the evaluation of semen quality. No definite criterion has been established for determining when sperm could be considered abnormal, and faulty techniques in making semen preparations for examination have a serious bearing on the problem. Sperm should be considered abnormal when their motility is affected, as this is the main essential for impregnation, also when their shape and structure digress from the normal so that motility is impaired, such as microcephalic sperm and those with bent tails.

Samples of ejaculated semen vary greatly in different bulls and in the same bull on successive ejaculations the same day or within short intervals of time. This is conditioned on the intensity of the orgasm, which reflects on the density of the ejaculate, concentration of sperm, motility, and longevity. Evidence would point to the fact that there are dead sperm in every ejaculate, even from perfectly normal, highly fertile bulls, and Anderson[1] considers that 70 per cent motility is the arbitrary, permissible limit for use in inseminations even though samples with a lower motility may prove fertile. Lagerlöf[10] stated that the number of pathologic sperm in a bull with good fertility does not exceed 17 or 18 per cent.

It must be recognized that abnormal sperm may result from temperature changes, manipulation in making the microscopic preparations, changes in tonicity of the solution, and so forth. Thus, curled tails can be produced by adding water,[20] bent tails by storage (fig. 7), and loose heads by clearing agents.[3]

Protoplasmic drops in ejaculated semen are usually present in a relatively few cells, less than 1 per cent. There is evidence that in very active service with an intense orgasm, sperm may be ejaculated from the epididymides and thus some sperm with drops may be considered quite normal. Branton and Salisbury[21] have shown 20 to 34 per cent to still remain on sperm cells in the ampullae. This we have confirmed, but we find wide variations (table 1). Lagerlöf[10] stated that a large number of these not fully ripened sperm occur both with a commencing degenerative process in the spermatogenic epithelium of the testicle and in a regeneration of the same.

Sperm containing the drop at the distal end of the connecting piece will show straight tails on ejaculation. If they are stored for one day or less at extremes of temperature (45 F.), a high percentage will develop a bent tail around the drop, and this seriously interferes with motility and presumably fertilizing ability. This bending of the tail will also be manifested in a certain percentage of cells without the protoplasmic drop (fig. 8). This is all evidence of the need for care in evaluating semen samples on morphology.

SUMMARY

This study has demonstrated that semen is best examined with the least manipulation both for scientific study as well as routine insemination work. It should be done as soon after collection as possible and preferably before cooling. Faulty methods of collection and techniques of handling semen are responsible for increasing the number of apparently abnormal sperm. In evaluation of semen quality, it should be recognized that in every

ejaculate from practically all bulls there is a percentage of dead sperm as well as deformed sperm. A clear understanding of what constitutes an abnormal sperm is of extreme importance. Simple methods of examination for sperm morphology have been described. With these techniques true abnormals were lower than generally reported. Sperm with protoplasmic drops were a constant feature of every ejaculate, and their presence in relatively low numbers (less than 1%) should be regarded as normal. The drop probably serves a nutritive function of the developing sperm. The appearance of bent-tailed sperm evidently results from too sudden exposure of semen to extremes of high or low temperature. The former may occur in the artificial vagina. The protoplasmic cap was found on a great majority of developing sperm, being most pronounced in the testis and epididymis, but also demonstrable on a small percentage of ejaculated sperm.

References

[1]Salisbury, G. W., Willett, E. L., and Seligman, J.: The Effect of the Method of Making Semen Smears upon the Number of Morphologically Abnormal Spermatozoa. J. Anim. Sci., *1*, (1942): 199-205.

[2]Lasley, J. F., Easley, G. T., and McKenzie, F. F.: A Staining Method for the Differentiation of Live and Dead Spermatozoa. Missouri Agric. Exper. Sta. Circ. 292, (1944): 1-8.

[3]Mercier, E., and Salisbury, G. W.: Effect of Techniques on Preparing Semen Smears for Staining on the Morphology of Bull Spermatozoa. J. Anim. Sci., *6*, (1947): 60-66.

[4]Anderson, J.: The Semen of Animals and Its Use for Artificial Insemination. Imp. Bur. Anim. Breed. & Gen., Oliver & Boyd, Ltd., Edinburgh, (1944): 1-151.

[5]Arey, L. B.: Developmental Anatomy. A Textbook and Laboratory Manual of Embryology. 4th Edition. W. B. Saunders & Co., Philadelphia, (1943): 1-612.

[6]Toothill, M. C., and Young, W. C.: The Time Consumed by Spermatozoa in Passing Through the Ductus Epididymides of the Guinea Pig as Determined by Means of India Ink Injections. Anat. Rec., *50*, (1931): 95-107.

[7]Merton, H.: Contributions on the Maturation of Sperm Cells. Anim. Breed. Abstr., *7*, (1939): 375.

[8]Lasley, J. F., and Bogart, R.: Comparative Study of Epididymal and Ejaculated Spermatozoa of the Boar. J. Anim. Sci., *3*, (1944): 360-370.

[9]Rodenz, E.: Versuch einer biologischen Morphologie des Nebenhodens. Archiv. f. Entwcklngsmechn. d. Organ., *103*, (1924): 593-628.

[10]Lagerlöf, N.: Sterility in Bulls, Vet. Rec., *48*, (1936): 1159-1171.

[11]Selivanova, O. A.: The Movement of and Disappearance of the Kinoplasmic Droplet in Spermatozoa from Farm Animals. Abstr. in Anim. Breed. Abstr. *7*, (1939): 180-181.

[12]Lagerlöf, N.: Morphologische Untersuchungen über Veränderungen im Spermabild und in den Hoden bei Bullen mit verminderter oder aufgehobener Fertilitat. Acta path. et microbiol. Scandinav. Suppl. XIX., Uppsala, (1934): 1-99.

[13]Gunn, R. M. C., Sanders, R. N., and Granger, W.: Studies in Fertility in Sheep. Austral. Coun. Scient. Industr. Res. Bull., *148*, (1942): 1-140.

[14]Baylor, M. R. B., Nalbandov, A., and Clark, G. L.: Electron Microscope Study of Sperm. Proc. Soc. Exptl. Biol. & Med., *54*, (1943): 229-232.

[15]Greenberg, B. E., and Garbill, S. L.: The Relation of Hyaluronidase in the Seminal Fluid to Fertility. Human Fertility, *11*, (1946): 1-2.

[16]Leonard, S. L., and Kurzrok, R.: A Study of Hyaluronidase Effects on the Follicle Cells of Ovulated Rat Ova. Endocrinol., *37*, (1945): 171-176.

[17]Cody, B. A.: Observations and Experiments Upon Spermatozoa of the Guinea Pig. J. Urol., *13*, (1925): 175-191.

[18]Popa, G. T.: A Contribution to the Biology of Spermatozoa. Proc. 2nd Int. Cong. Sex Res. Oliver and Boyd, London, (1930): 103-110.

[19]Marza, V.: Contributions to the Histochemistry of Sex Cells. Proc. 2nd Int. Congr. Sex Res. Oliver and Boyd, London, (1930): 96-102.

[20]Walton, A.: The Technique of Artificial Insemination (with an introductory chapter). Imp. Bur. Anim. Gen., Edinburgh. Oliver and Boyd, (1933): 1-56.

[21]Branton, C., and Salisbury, G. W.: Morphology of Spermatozoa from Different Levels of the Reproductive Tract of the Bull. J. Anim. Sci. *6*, (1947): 154-160.

The Placentome of the Cow

H. E. KINGMAN, D.V.S.
Cheyenne, Wyoming

RETAINED fetal membranes, calves born dead, abortion, and failure of implantation, can be traced directly to imperfect performance on the part of the placentome during some phase of its development.

The placentome is an organ specific to gestation and is nonexistent at any other time. It is a new growth. It harbors the placenta. It develops through the union of fetal and maternal tissues under the combined and integrated action of specific influences.

It is admitted that both the clinician and the biologist have reached a level of understanding where further progress is impeded by the extremely complicated and mysterious nature of the problems involved. As a result, it has become necessary to review the entire anatomy, histology, and physiology of the reproductive system, in regard to which the clinician is the final interpreter.

It is also admitted that in the past the clinician has viewed too superficially the problems of reproduction. He has been unduly influenced by such things as the membranes hanging from a cow or an aborted fetus, when he should have been thinking in terms of the physiology and pathology of the placentome.

On the other hand, the embryologist, the anatomist, the chemist, and the physiologist have been unable to provide the clinician with the answers that, when completed, will be extremely helpful.

With the clinical objectives constantly in mind, we shall approach the problems from the very beginning of gestation and proceed to lay a physiologic foundation, so far as knowledge of the subject will permit.

DEVELOPMENT OF THE PLACENTOME

Development and devolution of the placentome is characterized by well-defined phases or periods.

Phase One.—Phase one deals with the preparation of the uterus for the reception of the fertilized ovum. It follows estrum and includes the first thirty to fifty days of gestation. During this period, the fertilized ovum passes through the fallopian tube to the uterus without direct communication with the endometrium. It is obvious that many factors can enter into the success or failure of gestation. These can be of infectious, endocrine, nutritional, or hereditary origin.

Implantation can succeed only when the cells and tissues of the genital system have reached maturity, are in a receptive mood, and possess an adequate nutritional healthfulness.

Estrogenic and progesteronic changes can be produced artificially in the nongravid uterus, but the development of the maternal placentome requires something that we have not yet been able to supply.

Charles W. Hooker [1] has the following to say in regard to progesteronic influence upon the endometrium: "In the course of the study of the healing of uterine wounds, an apparently specific response to progesterone was observed in the mouse. The response consists of an enlargement and vesiculation of the nuclei of the cells of the endometrial stroma. Estrogens and androgens do not elicit this reaction."

In order to gain a better understanding of the physiology of the placentome, an enormous amount of investigation and experimentation has been conducted.

The preovulatory calf has been chosen as an experimental animal in order to study the species in which we are most interested. The experimental data and histologic picture must be dealt with very briefly at this time. The results of microscopic studies will be omitted. However, a few examples of macroscopic changes will be shown.

It is assumed that the genital tract of the calf at birth is unaffected by gonadal hormones.

Calf 159-45 and calf 158-45 were given 160 mg. of stilbesterol over a period of fourteen days. The vulvas exhibited the usual signs of estrum including flow of mucus.

Calf 158-45 was slaughtered two days after the last injection of stilbesterol and at the peak of estrogen stimulation. The caruncles were prominent with apices well defined and similar in appearance to those of an adult uterus.

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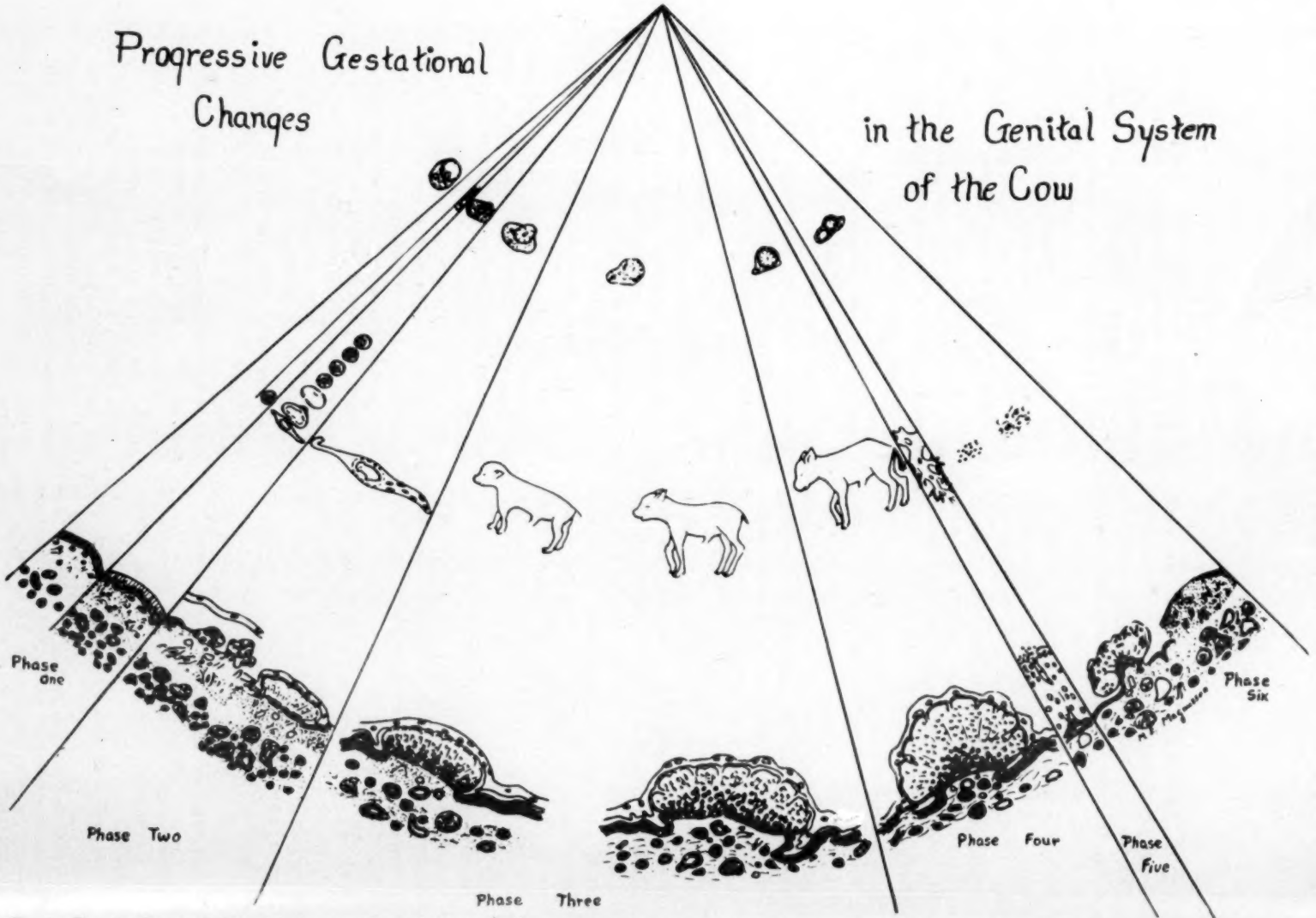

Fig. 1—The six phases of the genital system from the time of estrum through the period of gestation and the return of the genital system to a nongravid state.

The uterus of calf 159-45, slaughtered twenty-seven days following cessation of estrogen stimulation, had receded to a size that approximated that of the control calves in the experiment.

Roany, a 2-month-old calf, was injected subcutaneously with 125 mg. of progesterone over a period of twenty-five days and slaughtered one day after the last injection. The changes in the uterus are compared with the uterus of a 5-month-old control calf. The marked increase in the size of the uterine horn over that of the control will be noted.

The successful progressive development of the embryo is dependent upon the physiologic factors involved. It is the clinician's duty to familiarize himself with the details, so far as known, in order to handle the failures that are concomitant with this period. He takes cognizance of the following requisites:

1) A genetically sound sperm must have met an equally complete ovum.
2) United, they must have entered a genital tract that is anatomically and physiologically perfect.
3) Endocrine stimulation and inhibition must have been coördinated.
4) Nutritional support must have been adequate.

Phase Two.—This is a critical period. Many things can happen to interrupt gestation at this time.

Phase two, the fiftieth to the ninetieth day of gestation, is the period of implantation and includes those factors that make possible the first steps in establishing direct communication between the mother and the fetus. It is the stage in which the estrogenic influence is partially relinquished, and the progesteronic influence of the corpus luteum takes over the management of the genital system, not only of the maternal caruncles but of the fetal trophectoderm as well.

In this connection, Mosman [2] makes the following comment: "To really understand the implantation process, one must not only consider the preparation of the embryo but also that of the uterus itself, for implantation is carried out as a set of interactions between the embryo and the uterus. Neither from the physiological nor from the morphological standpoint can we logically separate behavior of the two parties in this process."

The processes of implantation of the chorion (which is an outgrowth of the trophectoderm) are initiated in phase two. It is the first actual appearance of the placentome. There is a definite roughness of caruncles in the region of the embryo, but during this period no macroscopic changes can be detected in the areas toward the ends of the horns. Thus, progress in development of placentomes is in a centrifugal direction from the embryo. This provides a splendid opportunity to observe the sequence of changes that take place in the endometrium, especially in the caruncles. (The term caruncle is used here to define a small eminence on the surface of the endometrium and from it, under certain stimuli, the placentome develops.)

By the ninetieth day of gestation, a fragile union has been established in the regions surrounding the embryo, but toward the apices the separation remains complete. This is a critical period in the life of the embryo. Its existence is easily terminated during this phase.

Phase Three.—Phase three is the period of evolution of the placentomes. The first placentomes to develop are in the region of the embryo and, from there towards the points of the horns, caruncles in turn form new placentomes as the requirements of the fetus demand. In this connection, the size of the fetus depends upon the area of the placental surface. Communication between the maternal and fetal tissues is fairly well established in the region surrounding the embryo by about the ninetieth day of gestation, and from there on to about the 230th day all of the placentomes within the uterus have reached, or nearly reached, maturity.

During this period, all of the functions that are essential to the growth and development of the fetus are dependent upon coördinated and intricate functions of the placentome. Some of the outstanding physical properties of the placentome should be noted. It is a new growth; it has many of the characteristics of a tumor; and it has no counterpart in the nongravid uterus. The caruncle from which it springs has none of the characteristics of the placentome itself. At first, it is a sessile area lying flat upon the surface of the endometrium, but traction on the part of the fetal membranes moves it away from the uterus and causes a neck to develop beneath the basal

Legends for Fig. 1 on Opposite Page

Phase 1—the preparation of the uterus for implantation (50 days).
Phase 2—the period of implantation (50 to 90 days).
Phase 3—evolution of the placentome (90 to 230 days).
Phase 4—the preparation of the genital system for parturition (230 to 285 days)
Phase 5—parturition.
Phase 6—puerperium; the return of the genital system to a nongravid state.

Fig. 1—The six phases of the genital system from the time of estrum through the period of gestation.

plate. The epithelium of the endometrium meets the placentome at its periphery and lines the entire surface provided by the maternal crypts.

The epithelium that covers each villus apposes that of the maternal crypt. There is an occasional bridge connecting these two epithelial surfaces but, as a whole, no direct contact appears to be maintained.

Phase Four.—Phase four (approximately the 230th to the 285th day of gestation) is that period during which the genital tract, including the placentome, ripens and becomes prepared for parturition.

The shock of parturition is cushioned by certain preparatory measures. Unfortunately, little is known of the factors that prepare the genital tract for the evacuation of products of conception. During this time, the physiologic phenomena change from those essential to growth of the fetus to those of preparation for its delivery. This cataclysmic event must be accomplished with the least possible injury to the fetus and mother.

A summary of the events taking place during this preparatory phase presents tremendous difficulties. This is due primarily to the paucity of substantial information. Investigators have recorded a marked interference with the blood supply to the fetus during the weeks prior to parturition. Bartholomew[3] states that: "In respect to vulnerability, lack of support from surrounding tissues, and subjection to direct trauma, the placental circulation is unique and unlike that of any other part of the body." Huggett[4] says, "After the thirty-second week (in the human, 224 days), obliterative endarteritis, placental infarction, and calcification are progressing, and the circulating volume of blood in the placenta decreases."

Endocrine readjustments essential to phase four are complex and poorly understood in spite of the effort that has been made to decipher them. Reynolds[5] states that, "estrin directly renders the myometrium irritable and contractile through the favorable metabolic conditions which it sets up within the tissues. In this, it brings about an increase of glycogen and of phosphocreatine, both of which substances are necessary for efficient muscular activity. In addition, glutathione and the calcium content of the uterus increase in concentration to a maximum."

Burrows[8] states: "The life of the placenta is dependent upon progesterone. Progesterone not only has an influence in the maintenance of pregnancy but plays an important part in the onset of parturition."

Reynolds[6] states: "It is possible, though by no means certain, that the withdrawal of placental influences which stabilize pregnancy is related to regenerative changes taking place in the placenta in the latter part of gestation where the uterus stops growing and embarrassment of uterine circulation ensues."

In previous discussions, quotations from different authors called attention to the fact that at the end of gestation (what we have termed phase four), the placenta is a senile organ. This is not apparent in the cow, but it is true that its function as an endocrine organ must be on the wane. One thing seems certain—the corpus luteum itself plays no part in phase four.

Edema of the posterior genital tract prior to parturition is not reflected in the placentome during this period. That edema plays an important rôle there can be little doubt.

Failure to complete the physiologic phenomena are common clinical experiences during the fourth phase. An understanding of the normal conditions is a prerequisite to the recognition of these failures and their prevention and treatment.

Phase Five.—The changes that take place in the placentome during parturition are of utmost importance. Here, the primary function of the placentome comes to an abrupt end. True, some preparation has been made for this cataclysmic ending, but the actual events concomitant with birth are distinctly separate from any other phase of gestation. Further, the events of phase five exert a determining influence upon the puerperal phenomena.

Like all living things, the placenta has an age cycle. The stroma of the villus becomes more cellular and compact as it grows older. The walls of the vessels become thicker, ultimately take on hyaline. The lumen becomes narrower and, in many villi, completely hyalinized and functionless. The umbilical vessels participate in infringement upon blood supply to the fetus and prepare themselves for rupture outside the umbilicus. Thus the placenta slowly ages and, near term, is a senile organ.

Reynolds[7] states: "Parturition begins as a result of the gradual accelerating conversion of a number of factors,—structural, humeral, nervous, nutritional, circulatory, which, at a time characteristic for each species and adapted to the morphological conditions present in each, are so associated that they lead to the evacuation of the uterus and contents."

One relevant fact, so far as the fetal placenta is concerned, is that its function as an intermediary organ completely ceases at parturition.

All of the cells are dead from the time of the rupture of the umbilical vessels. This is

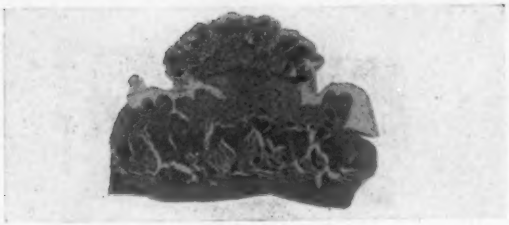

Fig. 2—Phase 1, the preparation of the uterus for implantation, about 50 days.

Fig. 3—Phase 3, the period of evolution of the placentome. (Early.)

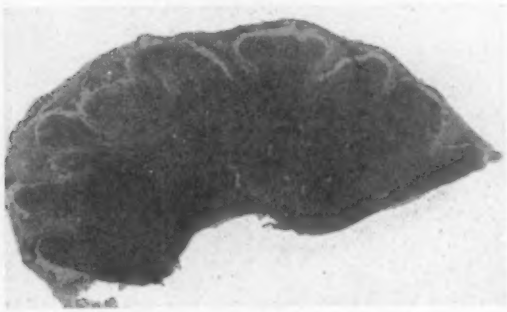

Fig. 4—Phase 3, the period of evolution of the placentome. (Late.)

Fig. 5—Phase 6, showing the involutionary, devolutionary, and reparative processes.

Fig. 6—Uterus (left) of calf 158-45, which was given 160 mg. of stilbesterol over a period of fourteen days. A biopsy of the uterus was obtained two days after the last injection of stilbesterol. At that time, the diameter of the horn, 1 in. from the bifurcation, was 14 mm.

Uterus (right) of calf 159-45, which was given 160 mg. of stilbesterol over a period of eighteen days and slaughtered twenty-seven days following the last injection of stilbesterol. The diameter of the horn at time of slaughter was 8 mm.

The process of photographing has resulted in an enlargement of almost 2 × (1.93%).

not true of the maternal tissue of the placentome. The maternal circulation, at least the large blood vessels, remain more or less intact. Accordingly, there is set up a sudden release of blood pressure from the fetal side as well as corresponding inhibition of circulation on the maternal side. There follows a uniform hemorrhage in the space that has existed between walls and the crypts and the fetal villus. This is a very important phenomenon. Convincing evidence is at hand to support the premise that the parturient placental blood clot governs, or at least influences, the release or adherence of fetal and maternal tissues.

In the cow, separation is facilitated by a certain amount of traction by the separated portion of the fetal membranes. Whether intervillus hemorrhage encourages separation has not been determined. Whichever comes first, hemorrhage or separation, may be the deciding point in interruption of the fetal and maternal relationships. At least the implications cannot be ignored.

Hemolysis takes care of the clot that remains in the maternal crypt following parturition. Evidence to support the belief that the blood clot occurs in the crypt of the cow lies in the assumption that the maternal cotyledon is a replica of the fetal cotyledon. We know that the fetal villi are always evenly covered by a tenacious clot. The only exceptions to this are those cases in which the clot has been removed by hemolysis and liquefaction or has not occurred at all, as in the case of some abortions.

The evidence that a maternal blood clot is being hemolyzed is seen in sanguineous lochia that follows parturition.

Summary of Phase Five.

1) Preparation for parturition has been made by the placentome in previous phases.
2) In spite of preparatory phenomena, the placentome is forced to absorb abrupt changes and reversals.
3) Infections, endocrine stimulations and inhibitions, nutritional provisions and deficiencies, and physical factors present at the time of parturition determine the course of resolution, involution, separation of fetal and maternal tissues, and devolution of the placentomes.
4) Intervillus hemorrhage is an important factor.
5) The umbilical vessels participate in preparation for parturition, and they occupy a vital position during parturition.
6) Interest at this time is directed toward the placentome. Other problems associated and related to parturition are left to the field of obstetrics.

Phase Six. — Phase six deals with the changes that take place in the genital system from the time the fetus is delivered until the genital tract has returned to normal.

It is made up of a number of concomitant and closely related events that are primarily separative, expulsive, and defensive; secondarily they are involutionary, devolutionary, and reparative. They take place in an orderly, effective, and integrated pattern that is suddenly accentuated by the initiated birth of fetus and the rupture of the umbilical vessels. These changes include the epithelial cells of the surface, the endometrial glands, the musculature, and lamina propria. The clinician must be familiar with the whole complicated series of events. He must be able to recognize the signs that are indicative of failure on the part of any tissue to perform its function effectively.

The separation of the fetal villi from the maternal crypts has long been a subject of investigation and speculation. Much misconception exists in regard to it, and the whole problem of separation or lack of it still remains little understood and totally unsolved.

Once the phenomena responsible for undue retention of fetal membranes have gone into action, "the die is cast." As a result, separation must take place by hemolysis and liquefaction. Until more is known of the forces involved in union and release, little can be done to affect them one way or another. The placentome is devoid of muscular tissue and therefore is not influenced by oxytocic agents. Devolution can not be hastened; perhaps it can be retarded. Repair can be supported by therapeutic agents such as estrogens, bismuth, and the manual removal of offensive debris.

Involution is seldom at fault. The ability of uterine muscle to respond to stimulation depends upon its condition. In the presence of acute inflammation, it must be allowed to rest. But in mild inflammatory processes, accompanied by relaxation of uterine muscle, stimulation of involution supports other physiologic postpartem phenomena.

CONCLUSION

Gestational phenomena in the cow follow six well-defined phases. Phase one (1-50 days) deals with the preparation of the uterus for reception of the fertilized ovum.

Phase two (50-90 days) is the period of implantation.

Phase three (90-230 days) is a direct continuation of preceding phases, set apart because certain phenomena undergo readjustment during this time.

Phase four (230-285 days) is that period in which the genital tract is prepared for parturition.

Phase five is concomitant with birth and the abrupt ending of the function of the placentome.

Phase six is the puerperal phase, from the time the fetus is delivered until the genital tract has returned to normal.

Pathologic conditions and clinical diseases occurring during, and peculiar to, these phases are death and resorption of the fertilized ovum, abortion and birth of dead calves, retained fetal membranes, and puerperal diseases (septicemia, pyometra, etc.). Clinicians and animal husbandmen have directed their attention to the superficial phenomena of infertility, abortion, and retained fetal membranes rather than upon the placentome itself.

References

[1]Hooker, Charles W.: A Criterion of Luteal Activity in the Mouse. Anat. Rec., *93*, (1945).

[2]Mosman, Harland W.: Comparative Morphogenesis of the Fetal Membranes and Accessory Uterine Structures. Contribution to Embryology, *26*, No. 158; 141.

[3]Bartholomew, R. A.: Pathology of the Placenta. J.Am.M.A., *3*, (1938): 2,276.

[4]Huggett, A. St. G.: The Nutrition of the Fetus. Physiologic Rev., *21*, (1941): 445.

[5]Reynolds, Samuel R. M.: Physiology of the Uterus. Paul B. Hoeber, Inc., New York, (1939): 303.

[6]*Ibid.*, 301.

[7]*Ibid.*, 298.

[8]Burrows, Harold: Biologic Action of Sex Hormones. Cambridge U. Press, London, (1945): 442.

Discussion

DR. GIBBONS (Ala.).—It was my privilege, starting in 1923, to do quite a lot of work with Dr. W. L. Williams on his study of the placenta of the cow. Some practitioners may wonder about the practical application of the particular thesis Dr. Kingman presented, but in his summary, he gave paramount thought to what Dr. Williams used to express, that the placenta is an absolute and graphic picture of the endometrium of the cow. I think any practitioner having a good dairy herd under his care should have the placenta examined after every parturition, whether the cow acts normally or not. Dr. Williams stressed that the placenta should be free from evidence of disease.

DR. GREEN (Ohio).—Would Dr. Kingman discuss the handling of retained placenta in the light of his findings?

DR. KINGMAN.—There would be nearly as many opinions on this matter as there are members present. I feel if there is an answer to be obtained to this specific problem, it may lie in a study of the circulation. We have progressed but little in the last twenty-five or thirty years so far as the handling of these problems is concerned. I am not saying we are not handling them skilfully.

I feel there is a place for the removal of fetal membranes, but that this should not be done routinely. The uterus should not be subjected to manual treatment for at least seventy-two hours postpartum. There are certain reactive and reparative processes going on within the basal part of the placentome and endometrium itself that are essential. It is doubtful that the removal of the fetal membranes facilitates the involutionary and beneficially reactive processes.

We have studied a great many sections taken during the different periods of evolution and devolution of the placentome. The evidence of reaction and repair is quite apparent. These natural protective forces must be supported and not embarrassed by being subjected to crushing the tissues and exposing susceptible areas. Attack the problem from within. Give sulfas by mouth or intravenously and intramuscular penicillin. Support the patient in her battle against invaders. Local protective agents will not reach far beneath the surface of the placentome or the endometrium, and it may be better to have both covered by fetal membranes than to denude them and lower the resistance of the tissues and animal by subjecting them and her to painful handling, along with the introduction of additional microörganisms.

On the Inheritance of Metatarsal Inclination in Ayrshire Cattle

ROBERT E. HABEL, D.V.M., M. Sc.
Ithaca, New York

THE CONFORMATION of the limbs of dairy cattle is a matter of considerable economic importance, especially in the purebred cattle industry. Certain deviations from an arbitrary norm have come to be regarded as faults which seriously handicap the animal in the show ring and decrease its sales value. The question of whether these deviations actually interfere materially with the efficiency of the dairy cow is highly debatable. Although conformation is often cited as a predisposing factor in the etiology of diseases of the joints, tendons, and hoofs, much of this reasoning is of the *post hoc, ergo propter hoc* variety. Many of the criteria of good limb conformation in cattle seem to be borrowed from the standard of excellence in the horse, where rapid gait and heavy draft produce forces of concussion and tension to which the bovine limb is not subject.

Nevertheless, as long as the faults in question are regarded as such by the industry, they will continue to present a real problem to the breeder. Any information which contributes to an understanding of the anatomic nature of the deviations, the objective comparison of individuals, or the heritability of limb conformation would be of substantial, practical interest. The present investigation of one phase of the problem was sponsored by the Ayrshire Breeders' Association.

REVIEW OF THE LITERATURE

The embryology and evolution of the skeleton of the extremities in mammals was described by Schmidt-Ehrenberg[27] and by Steiner.[31] They also discussed the homology of the bones of the tarsus and carpus. The special embryology of the extremities of Bos and Sus was described by Suschkind-Popowa.[33]

The anatomy of the bovine limb was treated by comparison with that of the horse in the textbooks of Ellenberger and Baum,[8] and Sisson and Grossman.[29] Schmidtchen[28] gave the extent of the tendon sheaths and bursae. It is apparent, from a review of the anatomic features which contribute to the maintenance of the angles of the joints of the hind limb, that the mechanism is extremely complex. The angle of each joint is dependent upon the others, and the number of factors involved is too great for individual evaluation by dissection.

Duerst[6] made an extensive study of the mechanics of support and locomotion in cattle. According to his analysis, the center of gravity shifted back and forth along the spine. In animals with long front limbs, in animals ascending a slope, in pregnancy, and when the paunch was filled, the weight shifted to the lower end of the spine because the viscera slid caudad on the floor of the abdomen. This was corrected by the muscle groups which straighten the angles of the hind limb, raising the caudal end of the spine. He observed that the heavy draft ox with straight limbs, while better supported, had a restricted stride and less thrust than the ox with more angulation.

According to Lake[16]: ". . . the posture of the body in general is maintained, not by the static tension of ligaments, but by the dynamic pull (postural tonus) of opposing muscles."

The score cards of the various dairy breeds[20] yield little concrete information as to the degree of angulation desired in the joints, in spite of the emphasis placed on this point in judging. Only the American Guernsey Cattle Club stipulates a nearly perpendicular metatarsus.

The angle of the stifle is the most important in the limb. The entire limb collapses if the muscles which fix the stifle are paralyzed. Duerst[6] gave the average angle of the stifle as 134 degrees 38 minutes for highland cattle, and 129 degrees for lowland cattle. These are taken from 738 cattle measured by himself and Behm.[3]

The most reliable estimates of the angle of the hock (tibiometatarsal) are Duerst's averages of 134 degrees for highland cattle and 129 degrees for lowland cattle. Pusch[23] and Grossbauer[11] stated that the tuber calcis is normally anterior to the transverse plane of the tuber ischii. Viewed from behind, the hock should be under the middle of the tuber ischii according to Pusch, and under the lateral tuberosity of the tuber ischii according to

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A thesis presented in partial fulfillment of the requirements for the degree of master of science, 1947, Ohio State University, Columbus.

Assistant professor of anatomy, New York State Veterinary College, Ithaca, N. Y.

Duerst. Grossbauer wrote that most cattle stand with the tuber calcis medial to the sagittal plane of the tuber ischii (cow-hocked). Pusch observed that this is true of all breeds except the modern (1895) English, the North German breeds with mixtures of English blood, and the Swiss purebreds. He listed the following faults:

1) Camped under — more or less sickle-hocked, tuber calcis far anterior to tuber ischii. This stance is conducive to inflammation of the bulbar corium.
2) Camped back — usually associated with straight stifle and hock, but may be also sickle-hocked if the tibia is long.
3) Straight limb—170 degree hock angle, serious in bulls, where the hock leverage is necessary in mounting for service.
4) Sickle-hocked—not rare in the lowland breeds, and especially in stabled cattle. In slight cases, the hock angle is 130 degrees to 135 degrees (Duerst's norm!). When the angle is as small as 120 degrees, the gait is affected with an awkward reaping (mähend) motion in advancing the foot. Marked sickle hocks only rarely appear at birth. The condition develops because of stabling and lack of exercise, and the consequent weakness of ligaments and tendons, especially when growth is retarded by poor feed or long periods of digestive disturbance. There is an inherited predisposition. The late maturing breeds (dairy and draft) have a long tibia, and although they may be camped back, they are also often sickle-hocked.
The increased tension of the gastrocnemius tendon in the sickle hock may cause exostosis of the tuber calcis, thickening of the tendon, or tendinitis. The angle of the fetlock may be changed by the pressure of the flexor tendons, and the digital axis may be broken.
5) Cow-hocked.
6) Bow-legged.

Pusch[23] adds a word of caution on judging conformation too severely:

> Das lange Unterschenkelbein ist im Verein mit der rückständigen Stellung und dem kleinen Sprungelenkwinkel unbedingt mehr hässlich als nachteilig, denn solche Ochsen haben stets einen geräumigen Schritt und solche Kühe sind oftmals recht gut Melker. Man darf deshalb in der Verurteilung der Stellung nicht zu weit gehen.*

Duerst made the statement that the angle of the hock is normally smaller in light animals, but that in heavy animals, camping under and sickle hocks only occur abnormally, usually as a *result* of hoof disease. Behm[3] remarked that it was generally known among breeders that constricted loin, sway back, and cow hocks were adaptations to a life of continuous stabling.

These authors have cited the following causes for a small angle of the hock: heredity of individual and of breed, stabling conditions, level lowland pasture, light weight, malnutrition, hoof malformation, and a long tibia.

The angle formed by the digit with the metatarsus was given by Duerst as 150 degrees to 160 degrees.

The relationship between the shape of the hoofs and the conformation of the limb has been mentioned. The question of whether the malformation of the hoofs causes the sickle-hocked conformation, or whether the reverse is true, merits some discussion. The literature on the bovine hoof comes mainly from Germany and Switzerland, where oxen are frequently shod, and dairy cattle are stabled under conditions which produce a high incidence of hoof malformation.

The normal angle formed by the dorsal wall of the hoof with the horizontal is 50 degrees in the forefeet, 55 degrees in the hind feet. The ratio of the height of the wall at the toe to the height at the heel is 2:1.[6] Grossbauer was in agreement, but said that the hind feet often have a more acute angle (47°), and the toe-heel ratio is commonly 4:3.

Grossbauer described the following mechanical changes in the bovine hoof at the moment of greatest weight bearing:

1) Expansion of the heels posterior to the line of greatest width of the foot. The expansion is greater at the coronet than at the ground.
2) Contraction of the half of the hoof which is dorsal to the greatest width, with a recession of the wall at the toe proximal to a 0.5-cm. indifferent margin on the ground.
3) Lowering of the distal interphalangeal (coffin) joint and the bulb.
4) Sinking of the sole.
5) Widening of the interdigital cleft, especially at the toes.

The anatomy and histology of the normal hoof and corium were described by Boas,[4] Eber,[7] Hohmann,[14] Hess,[13] Pohly,[21] and most exhaustively by Wyssmann.[35] One little known technical distinction should be noted in passing. It is the concensus of authorities that the true sole in the bovine hoof is restricted to a narrow crescent a few millimeters wide at the toe. The remainder of the ground surface is actually bulbar horn, homologous to the frog in the horse. This may explain the peculiar capacity for growth of the bovine sole.

The wide variety of malformations of the hoofs has been described under the general term *stallklauen*. In all cases, there is over-

*The long tibia, together with the backward placement of the leg, and the small angle of the hock undoubtedly combine to present an unsightly appearance or an unsoundness, for such oxen have a long, free stride and the cows are often good milkers. Therefore, one dare not judge posture too severely.

growth of the wall with elongation at the toe to 25 to 40 cm. The wall spreads on the abaxial side to a width of 10 to 20 cm. The ground surface of the hoof, which is normally 1 cm.

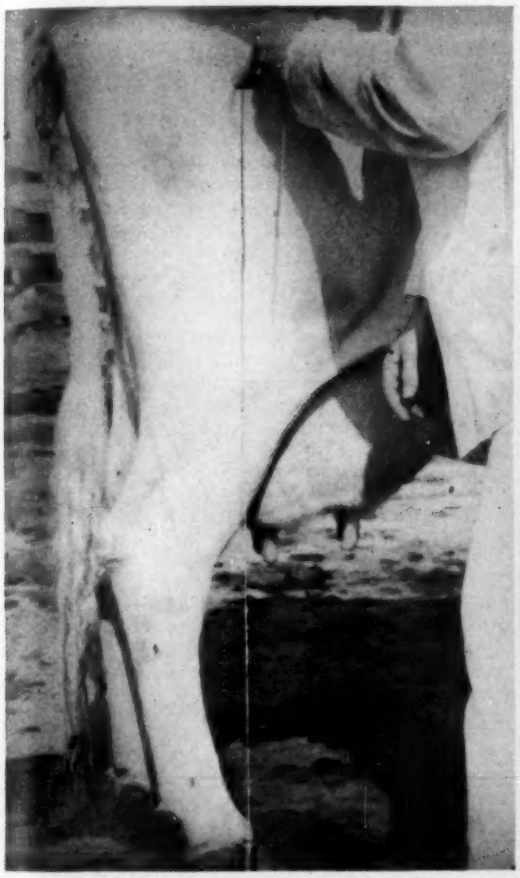

Fig. 1—The standard position of the limb. The plumb line is suspended from the trochanter major to the dorsal angle of the coronet. The metatarsal points are marked. (Reg. No. 222230, metatarsal inclination 70°).

thick, becomes 2.5 to 6 cm. thick under the toes. Often the horn inside the elongated toe disintegrates, leaving a cavity. Advanced cases may be crossed or recurved.

The etiology of bovine hoof malformations has not been determined experimentally. Two of the most authoritative writers on the subject, Pohly[21] and Fischer,[9] attributed the overgrowth entirely to lack of wear in animals standing continuously in soft, wet bedding. Pohly, however, published an illustration of abnormal hoof growth in a lame deer which showed as much overgrowth of the dew claws as there was in the third and fourth digits. This would indicate increased rate of horn growth, rather than a simple lack of wear. The dew claws in the normal animal do not reach the ground.

Another explanation is that the hoof malformation is caused by sickle hocks. The reasoning is that the inclination of the metatarsus and the camped-under position shift the weight from the toes to the heels. The toes are no longer in wear, and they become elongated as horn growth continues at the normal rate.[11,2]

Duerst was of the opinion that hoof overgrowth is hereditary, occurring in heavy, stabled cattle with soft horn which turns up at the toe instead of wearing off. He regarded black or red pigmented horn as harder than white. Sassen,[25] in experiments with equine

Fig. 2—Protractor aligned with metatarsal reference points.

hoofs, found that unpigmented hoofs were less resistant than pigmented. Hoofs from the same horse bearing equal pigmentation were equally resistant, but equally pigmented hoofs from different horses were not equally resistant.

Hess[13] stated that hoof malformation is a frequent sequel to laminitis in cattle. Dollar[5] observed that deformity from chronic laminitis was a rare condition.

One would expect to find a relationship be-

tween the nutritional status and the overgrowth of the hoofs, but the only references in this connection are to the rings formed by periods of malnutrition.

The pathologic changes produced by claw malformation were described by Pohly,[21] Fischer,[10] Schatzl,[26] and Hess.[13] The corium undergoes an acute to chronic inflammation as a result of pressure of the horn on the dorsal surface, crushing of the bulbs, and tension on the laminae at the abaxial surface. Exudate accumulates between the corium and horn on the abaxial surface, and separation may occur at the white line with consequent purulent cavitation.

The thick horn under the toe raises the tip of phalanx III 8 to 20 degrees from horizontal. Phalanx II becomes more vertical, so that the digital axis is broken. Fischer (fig. 34, 35[10]) showed radiographs of the foot before and after trimming the hoof, illustrating the change in the angle of phalanx III. Extensive changes occur in the internal architecture of the phalanges as a result of the altered lines of stress. Erosion of the articular surfaces may lead to ankylosis.

Fischer and Hess reported that the affected animals lie down much of the time. When standing, the hind limbs are camped under, and the angle of the hock is smaller (sickle-hocked).

All of the authors agree on the obvious treatment—regular hoof trimming. According to Hess,[13] it was necessary to remove 2 to 5 cm. of horn twice a year from the hoofs of cattle stabled continuously on soft standings. Fischer described the Allgau method used by professional trimmers. No mechanical means of restraint is used. The excess horn is removed from the ground margin of the wall with a hammer and chisel while the animal is standing on the foot. Then the toe is placed on a low block with the sole surface vertical, and the sole is shaved down with a wide knife, resembling a heavy plane blade with a "T" handle.

Behm[3] made an interesting comparative study of Central European cattle. He took 21 measurements on each of 600 animals of 17 breeds and computed averages for the highland and lowland types. The Brown Swiss is a highland breed familiar in this country, and although the Ayrshire was not studied by Behm, it may be taken as an example of a lowland breed.[22,32] Behm's observations are valuable descriptive data, whether or not one subscribes to his theory of "adaptation" to the terrain:

> Highland cattle graze on the side of a slope with the head relatively high. The neck is short. The center of gravity shifts caudad. The lumbar region sinks, especially in old animals, at pregnancy. The caudal end of the sacrum is elevated. The spine is curved with the convexity ventral. The tubera coxarum are pulled down by the weight of the viscera. The tubera ischiorum are elevated (*contra* Duerst). The hind limbs are camped back. All the angles of the limb are straightened. The sacrum, which is level and straight in calfhood, curves dorsad at the caudal end. The bodies of the second and third sacral vertebrae are wedge-shaped with the apices dorsal. The crista sacralis media is slender. The alae sacrales are long and thin. The anterior margin is thick; the posterior margin thin and sharp.
>
> Lowland cattle graze with the head very low. The neck is longer by an average difference of 13 cm. The back is arched. The center of gravity shifts craniad. The forelegs are camped under, throwing more weight on the shoulders. The scapula is longer by an average difference of 4.5 cm. from that of highland cattle. The hind legs carry less weight, but have more thrust in locomotion. They are camped under the body and the stifle and hock angles are smaller (sickle-hocked tendency). The sacrum is curved ventrad at the caudal end. The second sacral vertebra is wedge-shaped with the apex ventral, and appears to be crushed into the first sacral vertebra. The crista sacralis media is thick. The posterior margins of the alae sacrales are thicker than the anterior. As in highland cattle, the sacrum is straight and level in calfhood.

Pusch[23] noted similar differences between breeds. He observed that straight hocks and a short tibia were characteristics of early maturing (beef) and highland breeds.

Duerst[6] measured the progeny of lowland cattle imported to a highland district. The mean angles of the limbs in the progeny showed the following percentage differences from the means of the parents.

Stifle angle	+13.4%
Pelvis-femur angle	+ 7.15%
Declination of pelvis	+66.7%
Declination of sacrum	−66.7%
Shoulder angle	+ 8.0%
Increased height of rump over withers	+ 2.5 to 7 cm.

These results would suggest that the determination of the limb angles is largely environmental. It should be noted in this connection that the Brown Swiss has been grazing on lowland terrain in the United States for generations without undergoing changes at the rate indicated by Duerst's work.

Various hereditary limb deformities and lamenesses have been described by Duerst,[6] Mead,[18] Nielsen,[19] Van Drimmelen,[34] and Atkeson.[1] Most of these were semilethal recessives, and all were transmitted as simple Mendelian characters. They seemed to have little

relation to the present problem. Because of the large number of anatomic factors involved in limb angulation, and because the variation has a normal rather than a binomial distribution, it is appropriate to test the hypothesis of multiple factor inheritance.

METHODS

The animals measured in the present investigation were purebred Ayrshire cows in central Ohio herds. There was no deliberate random sampling. All of the milking cows of all the breeders who were willing to coöperate were included. Dry cows and heifers were not measured, except in rare cases when they were stanchioned with the others. The measurement of bulls was generally discouraged by the owners. Such results as were obtained from bulls were unreliable because of the difficulty in adjusting the limb under a psychologic handicap.

The cows were measured in the stanchions immediately after they had been milked. The skeletal reference points were marked with chalk as follows:

1) The anterior border of the trochanter major femoris. This marks the approximate plane of the acetabulum.

2) The tuberosity on the lateral surface of the proximal end of the metatarsal bone. This is the distal point of attachment of the long lateral ligament of the tarsus. The ligament is palpable as it extends from the malleolus fibulae to its attachment on the fibular and fourth tarsal bones, whence it turns distad to the metatarsal attachment.

3) The proximal attachment of the lateral deep collateral ligament of the fetlock joint. This is easily palpated from the lateroplantar tuberosity on the proximal end of the first phalanx, to the depression on the distal end of the lateral surface of the metatarsal bone.

When these three points had been determined, the limb was placed in a standard position so that a plumb line, suspended from the anterior border of the trochanter major, would intersect the coronet at the dorsal surface of the digit. The plumb line was actually used in every case.

With the limb in position, the inclination of the metatarsus was measured in degrees from the horizontal by means of a machinists's bevel protractor, a readily available substitute for the specially constructed clinometers used in hippometry. One arm of the protractor was aligned with the two points on the metatarsus; the other arm was leveled by means of the bubble, and the inclination was read from the scale.

Several features of this procedure are open to question. It may be argued that the measurement of one angle is not enough for evaluation of the conformation of the limb. This objection is quite valid. The method is not offered as a substitute for the judgment of experienced breeders. However, the inclination of the metatarsus is a function of all the other angles of the limb, and affords a simple, objective, quantitative, means of comparison. The actual tibiometatarsal angle was not used because it would have necessitated more skeletal reference points, which increase the probability of error. The points on the lateral surface were selected because the dorsal and plantar surfaces of the metatarsus are uneven and often curved, as a result of thickening of the tendons and fascia. The data collected for each subject were:

1) The name and registration number of the cow, sire, and dam.
2) Date of birth of the subject.
3) Last calving date.
4) Date of examination.
5) The anteroposterior inclination of the metatarsus as read from the protractor for right and left limbs.
6) An estimate of the position of the hock with respect to the tuber ischii, as viewed from the rear.
7) An estimate of the lateral inclination of the metatarsus.
8) An estimate of the amount of supination at the foot, in degrees from the median plane.
9) Notes on unusual circumstances, pathology of joints, overgrown hoofs, etc.

RESULTS

1) *Mean Inclination of the Metatarsus.*—The inclinations of the right and left metatarsus were averaged for each cow to give one measurement in degrees from the horizontal for each of 244 subjects. The range was 16.5 degrees (85.5° to 69°). The mean was 78.7 degrees, with variance 7.231, and standard deviation 2.689 degrees. The fiducial limits of the population mean at the 95 per cent probability level were 78.4 degrees and 79.08 degrees. This estimate of the population mean, and all of the estimates of parameters which are to follow, are applicable only to the population of Ayrshires in central Ohio, from which the sample was drawn.

2) *Regression of Metatarsal Inclination on Stage of Lactation.*—An attempt was made to demonstrate a regression of the size of the angle of inclination on the number of months since the last parturition. There was no significant regression.

3) *Regression of Metatarsal Inclination on Age.*—The range of the ages of the cows

measured was 14.5 years (16.5 years to 2 years). The median age was 4.5 years. There was a significant negative correlation ($r = -0.2673^{**}$) of the angle with the age. When this was computed as a regression coefficient, it was found that $b = -0.2834°/1$ year.** That is, the angle was more acute in the older cows by a difference of 0.2834 degrees per year.

The fiducial limits of b were: –0.1539 degrees and –0.4129 degrees. This regression cannot be extrapolated to animals younger than 2 years. Four measurements taken on calves were considerably smaller than expected. Perhaps the regression would be curvilinear if enough very young animals were measured.

The regression obtained in the present investigation could be interpreted in several ways: (a) the individuals may have developed a more acute angle as they became older; (b) intensive selection against the more acute angle may have changed the population so that the younger cows had a more desirable genotype; (c) culling for conformation in the younger cows measured may have been more intensive than in older cows which had established a production record; (d) some environmental factor may have changed over the period of time involved.

In order to test these possibilities, all of the coefficients of correlation to be discussed were computed once with the observed angle of inclination, and again with the data adjusted to the median age, 4.5 years.

The adjustment was accomplished by computing an expected angle ($\hat{Y}$) for each age (X).

(1)

$$\begin{aligned}\hat{Y} &= \bar{y} + b\,(X - \bar{x}) \\ &= 78.7 - 0.2834\,(X - 5.234) \\ &= 80.2 - 0.2834X\end{aligned}$$

From these values, a table of conversion factors was compiled, using a formula similar to that for mature equivalent milk production.[24]

(2)

$$\text{Adjusted angle (observed angle, Y)} = \frac{\hat{Y} \text{ at 4.5 yr.}}{\hat{Y} \text{ at age of cow, X}}$$

** The double asterisk is used in this report to indicate a highly significant statistic, *i.e.*, a value which would have less than 1 per cent probability of occurring in the sample by chance alone if the parameter were zero.

Every coefficient of correlation between relatives was higher when computed with observed angles. There was no difference between adjusted and observed data in the herd correlation.

TABLE I—Correlation Coefficients for Observed Angle and Angle Adjusted to Age 4.5 Years

Relationship	Correlation coefficient	
	Observed angle	Adjusted angle
Maternal half sister	0.20575	0.115
Paternal half sister	0.1665**	0.0728
Daughter-dam	0.4143**	0.143
Full sister	0.7630**	0.6687**

This effect of the adjustment on the correlation coefficients could only be attributed to (b) above. If any of the other possible explanations of the regression of angle on age were valid, the intraclass correlation between relatives would have been increased by the adjustment to a standard age.

Consequently, it has been assumed that the regression on age was largely attributed to a change in the genotype of the animals, and the remainder of the results to be discussed were based on the angle actually observed.

4) *Coefficient of Intraclass Correlation Among Maternal Half Sisters.* — There were 14 pairs of maternal half sisters, and one group of 3. The analysis of variance is given in table 2. (Snedecor,[30] p. 232.)

There was no significant variation between dam groups.

The intraclass correlation coefficient for groups of various sizes is computed by the formula: (Snedecor,[30] p. 245)

(3)

$$r_i = \frac{M_{\bar{x}} - M}{M_{\bar{x}} - (k_o - 1)M},$$

where $M_{\bar{x}}$ is the mean square for groups, M the mean square for individuals within groups, and k_o is an average of the number of individuals per group, computed by the formula: (Snedecor,[30] p. 234)

(4)

$$k_0 = \frac{1}{n-1}\left(Sk - \frac{Sk^2}{Sk}\right).$$

The intraclass correlation coefficient (r_I) for maternal half sisters was 0.2057. This is not significant in the strict sense because of the small value of F, but it cannot be ignored.

TABLE 2—Analysis of Variance of Metatarsal Inclination of 15 Groups of Maternal Half Sisters

Source of variation	Degrees of freedom	Sum of squares	Mean square
Between dam groups	n-1=14	124.8	8.9143 ($M_{\bar{x}}$)
Within groups	Sk-n=16	93	5.8125 (M)
Total	Sk-1=30	217.8	

$$F=\frac{M_{\bar{x}}}{M}=1.5336 \qquad F_{.05}=2.37$$

5) *Coefficient of Intraclass Correlation Among Paternal Half Sisters.*—The 43 groups of paternal half sisters varied in number per group (k) from 2 to 12. For this computation, it was necessary to eliminate the effect of full sisters by withdrawing k-1 of each group of full sisters.

TABLE 3—Analysis of Variance of Metatarsal Inclination of 43 Groups of Paternal Half-Sisters

Source of variation	Degrees of freedom	Sum of squares	Mean square
Between sire groups	n-1= 42	416.5	9.9166
Within groups	Sk-n=130	847.9	5.5233
Total	Sk-1=172	1264.4	

F=1.7957,** $F_{.01}40 \times 125.=1.75$, $r_I=0.1665$**

This correlation coefficient, although small, is highly significant because of the large number of animals involved (173).

6) *Coefficient of Intraclass Correlation Between Daughter and Dam.*—Table 4 shows that the coefficient correlation in 37 daughter-dam pairs was 0.4143.**

TABLE 4—Analysis of Variance of Metatarsal Inclination of 37 Daughter-Dam Pairs

Source of variation	Degrees of freedom	Sum of squares	Mean square
Between daughter-dam pairs	n-1=36	453	12.58
Within pairs	n(k-1)=37	192.84	5.21
Total	nk-1=73	645.84	

F=2.41,** $r_I=0.4143$,** $F_{.01}30 \times 36=2.26$

7) *Coefficient of Intraclass Correlation Among Full Sisters.*—Of the 13 groups of full sisters there were three groups of 3, and 10 pairs.

8) *Coefficient of Intraclass Correlation Among Herd Members Not Closely Related.*—As a means of estimating the effect of environment on metatarsal inclination, k-1 of each group of daughter and dam, full sisters, maternal half sisters, and paternal half sisters, were randomly drawn and removed from the sample, and the remaining data assembled in 14 herd groups. The groups varied from 2 to 20 members.

This result cannot be taken as conclusive evidence that environment had no effect. The removal of all the close relatives, although unavoidable, was a drastic measure which may have greatly increased the error. Another circumstance which makes this result unreliable is the fact that five of the 14

TABLE 5—Analysis of Variance of Metatarsal Inclination of 13 Groups of Full Sisters

Source of variation	Degrees of freedom	Sum of squares	Mean square
Between sister groups	n-1=12	138.84	11.57
Within groups	Sk-n=16	22.7	1.42
Total	Sk-1=28	161.54	

F=8.148,** $r_I=0.76298$,** $F_{.01}=3.55$

herds, including one of the largest, had been recently assembled. Therefore, differences of terrain or management had not had time to take effect.

It seems doubtful, however, that the metatarsal inclination as herein defined was influenced very much by environment. The standard position of the leg used in these measurements eliminated all of the variation caused by a camped-under stance. There is

TABLE 6—Analysis of Variance of Metatarsal Inclination of 14 Non-Sib, Non-Filial Herd Groups

Source of variation	Degrees of freedom	Sum of squares	Mean square
Between herds	n-1=13	105.01	8.078
Within herds	Sk-n=76	614.19	8.081
Total	Sk-1=89	719.20	

F=0, $r_I=0$.

little doubt, however, that such environmental factors as long, untrimmed hoofs, unsanitary standings, foot rot, and laminitis, are important in causing the cow to stand with her hind feet farther under the body. This gives a sickle-hocked appearance which is greatly improved when the hoofs are properly trimmed and treated.

The present data are not adequate for evaluating the relationship between length of hoof and metatarsal inclination, because few long hoofs were seen. Of 19 such cases, 12 had an angle of metatarsal inclination larger than the mean (78.7°), and 7 had a more acute angle, when measured in the standard position. This would indicate that the angle of the hock has little to do with the length of the hoofs.

DISCUSSION

The significance of the foregoing correlations can best be appreciated by comparing them with a theoretical standard, and with each other.

Assuming multiple factor inheritance and excluding the effects of selection, dominance, epistasis, inbreeding, and environment, the expected correlation for half sisters is 0.25, and for daughter-dam and full sisters, 0.5 (Lush,[17] p. 244).

In order to compare the observed correlations with each other and with the expected values, it is necessary to convert r_i, which has a skewed distribution, to z, which is distributed normally. This is accomplished by means of Fisher's[10] formula for the z transformation of an intraclass correlation coefficient in which k is greater than 2 (k_0 of Formula (4) was substituted for k).

(5)
$$z=\tfrac{1}{2}\{\log_e [1 + (k_0-1) r_i]-\log_e (1-r_i)\}$$

It is then possible to set fiducial limits at the 95 per cent probability level for each z, by interpolating the positive and negative deviations in Fisher's table VI. These values are set out in table 7.

TABLE 7—Comparison of the Observed Correlations.

Relationship	r_i	Expected r	z_{ri} with fiducial limits ()	z of expected r
Maternal half sister	0.2057	0.25	0.2139	0.2426
Paternal half sister	0.1665**	0.25	(0.5035) 0.2933 (0.1487)	0.4234
Daughter-dam	0.4143**	0.5	(0.731) 0.44 (0.1706)	0.549
Full sister	0.7630**	0.5	(1.512) 1.059 (0.6839)	0.549

It will be noted that none of the observed correlations, except that of full sisters, was significantly different from the expected value. The high full sister correlation suggests, in the absence of marked environmental influence, that there was a strong inbreeding or epistatic effect. The full sister correlation was also significantly higher than either the daughter-dam, or the doubled paternal half sister correlation (z_{2r_i} =0.49). It should be noted in connection with the interpretation of the full sister correlation, that it was taken from the smallest number of animals (Sk = 29).

Although the lower fiducial limit of z for daughter-dam correlation overlaps z for paternal half sisters, z for daughter-dam appears to fit z_{2r_i} =0.49 for doubled paternal half sister correlation very closely, as expected.

The heritability of a characteristic may be estimated roughly for a noninbred population as four times the half-sib correlation or twice the full-sib or daughter-dam correlation.[15, 12] The paternal half sister correlation would seem to be the best value to use for this estimate. It is based on more animals than the other correlations and is probably affected little by inbreeding. If a sire is used on cows from several different inbred families, of varying relationship to himself, his progeny will be more variable (smaller half-sib correlation) than in a noninbred population. The heritability of the metatarsal inclination, computed from the paternal half sister correlation was 0.666, a high value. With such a high heritability, one would expect to find that the characteristic was easily controlled by selection. The regression of angle on age, if accepted as evidence of genetic change, would support this view.

SUMMARY

An objective method for the measurement of metatarsal inclination in cattle is described. The mean inclination from the horizontal was 78.7 degrees for 244 cows varying in age from 2 years to 16 years. The coefficient of regression of the angle on age was −0.28 degrees** per year. Comparison of correlations between relatives, for observed data and for data adjusted for age, indicated that the regression was attributable to a genetic change in the population in the fourteen-year period. The maternal half sister intraclass correlation

in 15 groups was 0.2057, but was not significant. The paternal half sister correlation for 173 cows in 43 groups was 0.1665.** Daughter-dam correlation for 37 pairs was 0.4143.** Full sister correlation was 0.7630** in 13 groups. There was no correlation among unrelated members of the same herd. The heritability, estimated from the paternal half-sib correlation, was 0.666. It was concluded that the characteristic was highly heritable, and determined by multiple factor inheritance.

References

[1]Atkeson, F. W., Eldrige, F., and Ibsen, H. L.: Bowed Pastern in Jersey Cattle. J. Hered., *34*, (1943): 25-26.

[2]Bambauer, S.: Klauenkrankheiten des Rindes. Tierärztl. Rundschau, *44*. (1938): 381-384.

[3]Behm, H.: Untersuchungen über den Mechanismus der Hinter- und Vorderextremität des Rindes in seiner Verschiedenheit bei Tieflands und Höhenschlagen. Inaug. Diss., Bern. Schaper, Hannover, 1911.

[4]Boas, J. E. V.: Ein Beitrag zur Morphologie der Nägel, Krallen, Hufe, and Klauen der Säugetiere. Morphol. Jahrb., *9*, (1884): 389.

[5]Dollar, J. A. W.: Dollar's Veterinary Surgery, 3rd Ed. Baillière, Tindall, and Cox, London, 1945.

[6]Duerst, J. U.: Grundlagen der Rinderzucht. J. Springer, Berlin, 1931.

[7]Eber, A. L.: Beiträge zur vergleichenden Morphologie des Unpaarzeher- und Paarzeherfusses. Paul Parey, Berlin, 1895.

[8]Ellenberger, W., and Baum, H.: Handbuch der Vergleichenden Anatomie der Haustiere, 16th Ed. Julius Springer, Berlin, 1926.

[9]Fischer, A.: Das Klauenbeschneiden der Rinder. 7th Ed. Schaper, Hannover, 1934.

[10]Fisher, R. A.: Statistical Methods for Research Workers, 6th Ed. Oliver and Boyd, London, 1936.

[11]Grossbauer, J., and Habacher, F.: Der Huf- und Klauenbeschlag. Urban and Schwarzenberg, Berlin, 1928.

[12]Hazel, L. N., and Terrill, C. E.: Heritability of Weaning Weight and Staple Length in Range Rambouillet Ewes. J. Anim. Sci., *4*, (1945): 347-358.

[13]Hess, E.: Klauenkrankheiten, in Bayer-Fröhner Tierärztliche Chirurgie und Geburtshilfe. William Cranmüller, Vienna and Leipzig, Vol. *4*, Part 3. (1905): 117-352.

[14]Hohmann, Hugo: Untersuchungen über die Klauenlederhaut des Rindes. Monatsh. f. prakt. Tierh., *13*, (1901): 49-96.

[15]Knapp, B., Jr., and Nordskog, A. W.: Heritability of Growth and Efficiency in Beef Cattle. J. Anim. Sci., *5*, (1946): 62-70.

[16]Lake, N. C.: The Foot, 3rd Ed. Williams and Wilkins, Baltimore, 1943.

[17]Lush, Jay L.: Animal Breeding Plans, 3rd Ed. Iowa State College Press, Ames, 1945.

[18]Mead, S. W., Gregory, P. W., and Regan, W. M.: Hereditary Congenital Flexed Pasterns in Jersey Cattle. J. Hered., *34*, (1943): 367-372.

[19]Nielsen, J.: Undersogelser over forekomsten av arvelig lahmed inden for R. D. M. Nord. Jordbr. Forsk., *24*, (1942): 97-100.

[20]Petersen, W. E.: Dairy Science, Lippincott, Philadelphia, 1939.

[21]Pohly, W.: Die Stallklauen der Rinder. Arch. Tierheilk., *44*, (1918): 39-66.

[22]Prentice, Ezra P.: American Dairy Cattle. Harper and Bros., New York, 1942.

[23]Pusch, G.: Die Beurteilungslehre des Rindes. Paul Parey, Berlin, 1896.

[24]Ragsdale, A. C., *et al.*: The Relation Between Age and Fat Production in Dairy Cows. J. Dairy Sci., *7*, (1924): 189-196.

[25]Sassen, D.: Untersuchungen über die Widerstandsfähigkeit pigmentierten and unpigmentierten Hufhornes. Jahresb. Vet. Med., *65*, (1939): 345.

[26]Schatzel, E.: The Structure of Normal and Diseased Phalangeal Bones in Cattle. Wien. tierärztl. Monatschr., *23*, (1936): 645-646.

[27]Schmidt-Ehrenberg, Elisabeth C.: Die Embryogenese des Extremitätenskelettes der Säugetiere. Rev. Suisse de Zoölogie, *49*, (1942): 33-131.

[28]Schmidtchen, P.: Die Sehnenscheiden und Schleimbeutel der Gliedmassen des Rindes. Union Deutscher Verlagsgesellschaften, Stuttgart, 1906.

[29]Sisson, S., and Grossman, J. D.: The Anatomy of the Domestic Animals, 3rd Ed. Saunders, Philadelphia, 1940.

[30]Snedecor, G. W.: Statistical Methods, 4th Ed. Collegiate Press, Ames, Iowa, 1946.

[31]Steiner, H.: Der Aufbau des Säugetier-Carpus und Tarsus nach neueren embryologischen Untersuchungen. Rev. Suisse de Zoölogie, *49*, (1942). 217-223.

[32]Sturtevant, E. L., and Sturtevant, J. N.: The Dairy Cow, a Monograph on the Ayrshire Breed of Cattle. A. Williams, Boston, 1875.

[33]Suschkina-Popowa, N.: Die Ontogenese des Extremitätenskelettes von Sus und Bos. Bull. Soc. Imp. Nat. Moscou, N. S., *28*, (1914): 209-278.

[34]Van Drimmelen, G. C.: Lameness in a Stud Cattle Herd, Possibly of Hereditary Origin. J. South African Vet. M. A., *13*, (1942): 105-110.

[35]Wyssmann, E.: Zur Anatomie der Klauenlederhaut. Arch. f. wissensch, prakt. Tierheilk, *28*, (1902): 577.

** Indicates a highly significant statistic.

Penicillin Therapy in Chronic Bovine Mastitis. I. Sensitivity of Bovine Mastitis Organisms to the Action of Penicillin

R. ALLEN PACKER, D.V.M., Ph.D.
Ames, Iowa

PENICILLIN has been proved one of the most important drugs used to combat certain bacterial infections in man. In some cases, the improvement and recovery of the patient have been spectacular. Because of the close similarity of some infections in animals to those in man, it was at once reasoned that disease conditions in animals caused by similar types of microörganisms also might be treated successfully with penicillin.

Streptococci and staphylococci are susceptible to the action of penicillin. Since the etiologic agent in a majority of cases of bovine mastitis is either a Streptococcus or a Staphylococcus, it was believed that penicillin would undoubtedly be effective in this important disease of dairy cattle.

Numerous reports of the clinical use of penicillin in bovine mastitis have been favorable. These reports have usually discussed only the clinical recovery or the successful elimination of the invading organisms from the infected udder. Each report records a number of unsuccessful treatments but gives no explanation of the failures. Some of the possible explanations of failure of penicillin therapy are: (1) the occurrence of naturally resistant organisms; (2) the development of penicillin resistance of the organism during treatment. and (3) the failure to maintain adequate levels of the drug in the udder for a sufficient length of time.

The value of any chemotherapeutic agent is determined by the sensitivity of the causal agents to the drug, by the amount of the drug which may be maintained in susceptible tissue, and by the actual results of controlled treatments. Consequently, this study is divided into three parts: Part I, the sensitivity of bovine mastitis organisms to the action of penicillin; Part II, penicillin levels in the udder during treatment; Part III, the treatment of cases of mastitis with penicillin.

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Part I

LITERATURE

A few reports on the sensitivity of mastitis organisms to penicillin have appeared thus far in the literature. Foley[2] tested a number of mastitis organisms and found *Staphylococcus aureus* to be inhibited by 0.02 to 0.15 Florey units/cc. of medium, *Streptococcus agalactiae* by 0.02 to 0.17 units, and *Streptococcus dysgalactiae* by 0.02 units. Kakavas and Scott[5] reported on a particular strain of *Str. dysgalactiae* which was sensitive to 0.006 to 0.008 Oxford units/cc. of medium. Seeley, Anderson, and Plastridge[11] tested a number of species of bacteria and found *Sta. aureus* strains sensitive to 0.03 to 0.09 O.U./cc. of medium, *Str. agalactiae* 0.03 to 0.18 units, *Str. dysgalactiae* 0.016 to 0.03 units, and *Streptococcus uberis* 0.03 to 0.36 units/cc. Murane[8] found that both staphylococci and streptococci grew in 0.01 O.U. but were inhibited by 0.1 O.U. Watson[12] tested one strain of mastitis Streptococcus, group C, which had a penicillin sen-

TABLE 1—Comparison of Penicillin Sensitivity of Mastitis Organisms

		No. of cultures tested	Greatest concentration of penicillin per cc. in which growth occurred							
			10	5	1.0	0.5	0.25	0.125	0.06	0.031	0.015
Sta. aureus	A	105	1	5				1	44	51	4
	B	32	1	1	1		2	3	15	8	1
Str. uberis	A	10							2	6	2
	B	15						2	3	7	3
Str. dysgalactiae	A	19							4	6	9
	B	2									2
Str. agalactiae	A	33*							22	24	7

A=Cultures from herds in various parts of Iowa.
B=Cultures from Iowa State College herd.
*=Eleven of these strains furnished by Dr. O. W. Schalm, U. of California, Berkeley.

sitivity of 0.0156 O.U./cc. of medium. Heishman[4] tested 53 strains of *Str. agalactiae* and 22 strains of *Str. uberis* and found their sensitivity range between 0.031 and 0.125 units/cc. Three strains of *Str. dysgalactiae* were sensitive to less than 0.031 units. Fifty-one strains of *Sta. aureus* had a sensitivity of 0.062 to 0.125 units, while one strain of this species grew well in the presence of 2 units/cc. Numerous reports of successful treatment of streptococcic and staphylococcic mastitis would also indicate that these organisms are sensitive to this drug.

Methods of Procedure and Results

A majority of the organisms were tested by using a streak plate method. The various organisms were isolated from milk samples and were maintained on tryptose agar slants until they were tested. The time between isolation and testing varied from two days to four months. The technique of testing was as follows: A suspension of the organism to be tested was made in 1 cc. of sterile saline by removing a portion of the growth from an 18-hour agar slant culture. An amount of growth sufficient to make a turbidity to match tube 1 of the McFarland nephelometer scale was used. A loopful of this suspension was streaked on a tryptose blood agar plate containing penicillin. Various concentrations of penicillin were incorporated in the blood agar by adding the required amount of penicillin to the medium at a temperature of 45 to 48 C. just before it was poured into the plates. A regular, commercial grade of sodium penicillin* in 100,000 O.U. vials was used as the standard penicillin and was found to be quite uniform in potency. Four different organisms were tested per plate by dividing the plate into four sections. After inoculation, the plates were incubated at 37 C. for forty-eight hours, at which time readings were made. The presence or absence of growth was recorded and the amount of growth compared with that on a control blood agar plate containing no penicillin. The control culture also served to check the purity and viability of the inoculum used. A test strain of *Sta. aureus*, 313,** was tested with each group of organisms to check variations in different batches of plates that were prepared. Each culture, with few exceptions, was tested at least twice, using the technique described.

A few tests were made using tryptose broth in tubes instead of blood agar plates; the remainder of the procedure was the same as that described for blood agar plates.

*Furnished by Merck and Co., Inc., Rahway, N. J.
**Courtesy L. O. Krampitz, Department of Bacteriology, Iowa State College, Ames.

A number of trials were made to determine the bactericidal sensitivity of representative organisms. Starting with either sterile skimmed milk or tryptose broth, serial dilutions of sodium penicillin were made to give final concentrations varying from 0.015 to 1,000 O.U. /cc. of medium. One drop from a 1-cc. pipette of a 24-hour broth culture was used as the inoculum. These tubes containing the penicillin and organisms were incubated at 37 C. for forty-eight hours and observed for the presence or absence of growth. They were then subcultured in broth to detect the presence of viable organisms.

In table 1, the penicillin sensitivity of the various species of mastitis organisms as well as the strains within the same species are compared. These organisms likewise are divided as to source. Those which were isolated from the Iowa State College herd are compared with strains isolated from milk samples sent to the laboratory from dairy herds throughout Iowa. It will be noted that there are no significant differences in penicillin sensitivity between the two sources named. A majority of the mastitis organisms were completely inhibited by 0.125 O.U. or less. It is significant that resistant strains of *Sta. aureus* appeared in the college herd as well as other herds. This characteristic of comparative penicillin resistance is significant with respect to treatment, as will be shown later.

TABLE 2—Comparison of the Sensitivity to Penicillin Before and After Treatment in Cases which did not Respond to Treatment

Culture	Species	Sensitivity before treatment (O.U.)	Sensitivity following treatment (O.U.)
2524—2	*Sta. aureus*	.06—.125	.06—.125
2061—2	*Sta. aureus*	.06—.125	.03—.06
2061—3	*Sta. aureus*	.06—.125	.06—.125
1839—1	*Sta. aureus*	.06—.125	.03—.06
2019—4	*Sta. aureus*	.06—.125	.06—.125
1828—1	*Sta. aureus*	.03—.06	.03—.06
1828—2	*Sta. aureus*	.03—.06	.06—.125
1725—3	*Sta. aureus*	.03—.06	.06—.125
2238—1	*Sta. aureus*	.06—.125	.06—.125
1977—2	*Sta. aureus*	2—5	2—5
2359—1	*Str. uberis*	.015—.03	.015—.03
2500—1	*Str. uberis*	.03—.06	.03—.06

Table 2 shows the sensitivity of a number of organisms before and after penicillin therapy where the treatment was unsuccessful. No significant changes in susceptibility to penicillin were found among those tested. All of the various strains of staphylococci were uni-

formly sensitive to 0.05 to 0.125 O.U. of penicillin, with the exception of strain 1977-2, which was notably resistant to as much as 5 O.U./cc. of medium.

In table 3, there is practically no difference between the penicillin sensitivity of these eight organisms determined in liquid compared to solid medium. This is similar to the finding of Foster and Woodruff[3] who found no difference in agar plate and serial dilution methods of testing. There are significant differences between the bacteriostatic and the bactericidal effect of penicillin on the same strain. It takes considerably less penicillin to inhibit bacterial growth than it does to kill that same organism. There is more difference between the bactericidal amount and the bacteriostatic concentrations of penicillin for staphylococci than for streptococci. The bacteriostatic concentration for either organism is near 0.03 to 0.06 O.U. /cc. while the bactericidal concentration for staphylococci is about five to six times greater than that for streptococci. Seeley, Anderson, and Plastridge[11] have reported that the lethal amount of penicillin for *Sta. aureus* was higher than the amount required to inhibit growth. They found that about 49 O.U. were required to kill *Sta. aureus* in two days. The figures given in table 3 range from 7 to 50 units for *Sta. aureus*.

DISCUSSION OF RESULTS

In general, the staphylococci and streptococci which are associated with bovine mastitis are quite susceptible to the inhibitory action of penicillin. Only 16 out of 236 cultures of mastitis streptococci and staphylococci studied were able to grow in the presence of 0.125 O.U. All of the remaining 220 were completely inhibited by this concentration. These may be compared with other organisms tested at the same time but not included in table 1, such as *Brucella abortus* which grew in mediums containing 5 to 10 O.U.; *Pasteurella multocida* which grew in 0.25 to 0.5 O.U.; *Salmonella pullorum* and *Proteus ammoniae* which grew abundantly in the presence of 10 O.U.

There were no resistant strains of streptococci found, and there were no marked differences in penicillin sensitivity among the three species of mastitis streptococci. The only difference was with *Str. dysgalactiae* in which more of the strains were inhibited by 0.06 O.U. than the other two species. It would appear that *Str. dysgalactiae* is slightly more susceptible than *Str. uberis* or *Str. agalactiae*. Forty-two strains of *Str. agalactiae* isolated from Iowa dairy cattle were compared with 11 strains isolated in California. There were no differences in sensitivity in the strains isolated from these two places.

These findings are in agreement with those reported in the literature already cited [2,5,7,11] except for the resistant strains of *Sta. aureus* which have been mentioned. Heishman[4] is the only investigator reporting resistant strains of *Sta. aureus*. He found one strain among the 53 he tested which grew in 2 units of penicillin. This strain is very similar to the eight strains reported in table 1 which grew in 1 to 10 units per cubic centimeter. These eight resistant strains were otherwise typical of *Sta. aureus* by being hemolytic and by coagulating rabbit plasma. They were isolated from typical cases of chronic mastitis.

The penicillin sensitivity of two staphylococci was observed to remain quite constant

TABLE 3—Comparison of the Penicillin Sensitivity by Agar Plate and Tryptose Broth Methods and Bacteriostatic vs. Bactericidal Actions of the Drug

Culture	Species	Bacteriostatic action of penicillin		Bactericidal action in broth or skim milk (O.U.)
		Solid medium (O.U.)	Liquid medium (O.U.)	
2524—1	*Sta. aureus*	.06—.125	.06—.125	7—15
1839—1	*Sta. aureus*	.03—.06	.06—.125	15—30
1828—1	*Sta. aureus*	.03—.06	.03—.06	25—50
1828—4	*Sta. aureus*	.06—.125	.06—.125	25—50
2383—3	*Sta. aureus*	.06—.125	.06—.125	25—50
2383—4	*Sta. aureus*	.06—.125	.06—.125	25—50
2359—4	*Str. uberis*	.06—.125		3— 6
H —26	*Str. zoöepidemicus*	.03	.007—.015	3— 6

over a period of time in two instances. The test strain of *Sta. aureus*, 313, was maintained on artificial medium for over two years and was tested repeatedly during that period. The other instance was case 2371 in which *Sta. aureus* was isolated and tested and, after fourteen months, reisolated and tested again. In neither case was there any difference in penicillin sensitivity.

Although penicillin resistance can be produced by placing an organism in contact with sublethal concentrations of penicillin,[9] none of the strains of resistant *Sta. aureus* shown in table 1 had any history of penicillin therapy. They were naturally resistant strains.

It is significant to note from table 2 that "penicillin fastness" did not occur in any of the cases which did not respond to penicillin therapy. There was no appreciable change in the sensitivity of the organisms isolated before and after penicillin treatment. Failure to eliminate the etiologic agent in these cases was not due to natural or acquired resistance of the organism but to other factors. This observation confirms that of Murnane[7] who tested eight strains of *Sta. aureus* before and after treatment and found no change in sensitivity.

As a general rule, antibacterial agents which kill microörganisms are able to inhibit growth in lesser concentrations, which applies to penicillin as is shown by table 3. The amount of penicillin necessary to kill *Sta. aureus* in forty-eight hours is over 100 times the amount required to inhibit the growth of that organism. This difference is not as great with the two species of streptococci tested, which suggests that streptococci are comparatively more easily killed than staphylococci, while the inhibitory levels are more nearly the same. If successful therapy depends on a lethal effect, higher levels of penicillin would be necessary to successfully treat a case of staphylococcic mastitis than one infected by streptococci. Clinical experience reported in Part III of this paper, as well as a number of other reports,[1,6,7,8,10] tends to support this view. Perhaps the bactericidal sensitivity of an organism is a better index for predicting successful treatment with penicillin than the inhibitory sensitivity.

References

[1]Byrne, J. L., Pullin, J. W., and Konsk, H.: Studies in Infectious Mastitis. I. Penicillin as a Therapeutic Agent. Canad. J. Comp. Med. and Vet. Sci., *10*, (1946): 16-23.

[2]Foley, E. J., Lee, S. W., and Epstein, J. A.: The Effect of Penicillin on Staphylococci and Streptococci Commonly Associated with Bovine Mastitis. J. Milk Tech., *8*, (1945): 129-133.

[3]Foster, J. W., and Woodruff, H. B.: Microbiological Aspects of Penicillin. I. Methods of Assay. J. Bact., *46*, (1945): 187-202.

[4]Heishman, J. O.: Sensitivity to Penicillin of Microörganisms Associated with Bovine Mastitis, Am. J. Vet. Res., *8*, (1947): 257-259.

[5]Kakavas, J. C., and Scott, E. G.: Pencillin Blood-Level Determinations with a *Streptococcus dysgalactiae* Resistant to Normal Blood Inhibitors. Science, *104*, (1946): 327-328.

[6]McCulloch, E. C.: The Resistance of Chronic Staphylococcic Bovine Mastitis to Massive Penicillin Therapy. Am. J. Vet. Res., *8*, (1947): 173-177.

[7]Murnane, D.: A Preliminary Report on the Treatment of Clinical and Sub-clinical Streptococcal and Staphylococcal Infections of the Bovine Udder with Penicillin. Austral. Vet. J., *21*, (1945): 82-90.

[8]Murnane, D.: Second Report on the Treatment of Clinical Streptococcal and Staphylococcal Infections of the Bovine Udder with Penicillin. Austral. Vet. J., *22*, (1946): 35-40.

[9]Rammelkamp, C. H., and Maxon, T.: Resistance of *Staphylococcus aureus* to the Action of Penicillin. Proc. Soc. Exptl. Biol. and Med., *51*, (1942): 386-389.

[10]Schalm, O. W., and Little, R. B.: Treatment of Masitis *in*, Little, R. B., and Plastridge, W. N.: "Bovine Mastitis." McGraw-Hill Book Co., New York, (1946): 395-409.

[11]Seeley, H. W., Anderson, E. O., and Plastridge, W. N.: Action of Penicillin against Mastitis Organisms in Milk. J. Dairy Sci., *28*, (1945): 887-891.

[12]Watson, R. F.: Sensitivity of Various Serological (Lancefield) Groups of Streptococci to Penicillin. Proc. Soc. Exptl. Biol. and Med., *57*, (1944): 65-69.

The Permeability of the Lactating Bovine Mammary Gland to Sulfonamides

V. T. SCHUHARDT, Ph.D., T. B. CARROLL, D.V.M., L. J. RODE, M. A., AND HELEN LACY, B.A., M.T.

Austin, Texas

ALTHOUGH cattle have been treated for a variety of diseases with sulfonamides, there is little evidence in the literature relative to the permeability of the lactating bovine mammary gland to these drugs. Bauer and Gunderson[2] studied the blood and milk concentrations of sulfanilamide in a cow fed 1 oz. of the drug daily for twenty-two days. The highest milk concentration noted by these workers was 0.7 mg./100 cc. Several workers, Adair, Hesseltine, and Hac,[1] Stewart and Pratt,[6] and Pinto,[4] have reported on the permeability of the lactating human mammary gland to sulfanilamide. All of these workers reported milk concentrations equal to or exceeding the blood concentration of the drug.

In an experiment designed to test the effect of sulfadiazine on bovine brucellosis, Schuhardt, Rich, and Beal (unpublished) fed three groups of 6 cows each 1, 1.5, or 2 oz. of the drug per cow twice daily for a period of ten days. Some of the cows failed to eat all of the drug-feed mixture, but this failure did not correlate with the amount of drug administered. Free sulfadiazine concentrations were determined daily on milk from each of the test cows. These milk concentrations of the drug were compared with the blood serum concentrations on the second day, and with whole blood concentrations on the seventh and tenth days of the experiment. All sulfadiazine determinations were done by the Bratton and Marshall[3] colorimetric technique.

Table 1 records a comparison of the blood and milk concentrations of free sulfadiazine on the three occasions when both were tested. Rarely did the milk concentrations exceed 3.0 mg./100 cc., whereas the blood concentrations ranged up to 32.4 mg./100 cc. These results emphasize the significance of a tissue permeability factor as a consequence of which failure to cure a mammary gland infection might be explained in terms of the relative impermeability of the gland to the drug used.

TABLE 1—Comparison of Milk and Blood Concentrations of Free Sulfadiazine in Cows

		Milligrams of sulfadiazine/100 cc.					
	Drug	Second day		Seventh day		Tenth day**	
Cow No.	/day* (oz.)	Blood serum	Milk	Whole blood	Milk	Whole blood	Milk
41	2	5.2	1.6	8.0	2.2	5.8	2.4
88	2	4.8	0.8	8.2	1.2	6.2	2.4
96	2	6.5	1.4	6.1	0.8	5.0	2.2
97	2	10.0	1.6	8.9	1.8	6.6	2.4
99	2	4.4	1.3	7.9	1.4	4.8	2.2
153	2	4.7	1.6	8.0	1.4	7.6	3.4
42	3	8.3	2.2	8.2	2.6	3.8	3.0
78	3	5.7	1.6	11.2	2.2	5.9	2.8
84	3	6.8	2.1	12.4	2.2	7.4	1.8
93	3	4.6	1.6	7.6	1.4	5.7	3.2
102	3	3.9	1.0	5.6	0.4	3.2	2.3
105	3	6.7	1.3	9.0	1.4	1.6	0.8
82	4	8.3	2.1	11.3	3.4	12.8	5.6
89	4	9.2	1.6	27.6	3.0	17.0	3.1
100	4	8.6	1.9	12.0	2.8	11.6	2.2
101	4	5.4	1.4	32.4	2.8	12.0	2.5
115	4	8.5	2.2	12.4	2.0	18.6	3.0
155	4	7.7	1.9	7.6	0.6	5.9	1.2

*One half these amounts was mixed with the feed twice daily for ten days.

**Six hours after the last dose of drug.

EXPERIMENTAL

To test this tissue factor more extensively, it was decided to test the permeability of the lactating bovine mammary gland to eight sulfonamides: sulfanilamide, sulfamerazine, sulfathiazole, sulfapyrazine, sulfapyridine, sulfadiazine, 2-sulfanilamido-5-bromo pyrimidine, and 2-sulfanilamido-5-chloro pyrimidine.

Eighteen Jersey cows, selected on the basis of approximately equal size and milk production, were used in two experiments with these drugs. In the preliminary experiment, each drug was administered to a single cow. In the repetition of the experiment, each drug was administered to a second cow with the exception of sulfapyridine which was administered to 3 additional cows. Each cow received 90 Gm. of the appropriate powdered

From the Brucellosis Research Project of the Clayton Foundation and the University of Texas, Austin.

Professor of bacteriology and director of the Clayton Foundation Brucellosis Research Project (Schuhardt); veterinarian of the Texas State Board of Control (Carroll); bacteriologist of the Clayton Foundation Brucellosis Research Project (Rode); assistant bacteriologist of the Clayton Foundation Brucellosis Research Project (Lacy).

drug in capsules by mouth in three equal doses of 30 Gm. each at intervals of four hours.

Milk samples from the four quarters of the udder were collected from each cow before the drug was administered. Both blood and milk samples were collected from each cow at six, ten, twenty-two, thirty-four, and forty-six hours after beginning treatment. All blood and milk samples were tested for sulfonamide concentrations by the colorimetric technique of Bratton and Marshall.[3]

Table 2 lists the free sulfonamide concentrations observed in each cow. The blood levels of the eight drugs reached maximum concentrations somewhere in the neighborhood of twenty-two hours after the first dose (fourteen hours after the last dose). The significance of this observation to the timing of doses for maintaining therapeutic concentrations in cows is obvious.

Only two of the drugs failed to approach blood concentrations of 5 mg./100 cc. or >. These were sulfathiazole (1.8 mg./100 cc.) and sulfapyrazine (2.9 mg./100 cc.), and neither of these was demonstrable in the free form in the milk. However, three other drugs which reached good to excellent blood levels, sulfadiazine, and the bromo and chloro pyrimidines, failed to penetrate the mammary gland into the milk (sulfadiazine came through in 1 cow at a milk concentration of 1.1 mg./100 cc.). Sulfamerazine came through into the milk of each cow receiving this drug, but the maximum milk concentrations (2.6 and 1.5 mg./100 cc.) did not approach those reached in the blood (10.2 and 6.7 mg./100 cc.).

In contrast to this failure of six of the sulfonamides to permeate the mammary gland in therapeutic concentrations (5± mg./100 cc.), sulfanilamide and sulfapyridine reached concentrations of the free drugs in the milk approximating those found in the blood. Of the two, sulfapyridine reached higher concentrations of the free drug in both blood (7.2 to 10.6 mg./100 cc.) and milk (5.8 to 7.4 mg./100 cc.).

When free and total sulfonamides were compared (table 3) in the twenty-two-, thirty-four-, and forty-six-hour samples of both blood and milk, it became apparent that some of the acetylated form of each of the drugs appeared in the milk. Sulfanilamide is particularly noticeable in this regard. Total sulfanilamide levels ranged from 13.8 to 17.6 mg./100 cc. in the milk, whereas, the total sulfanilamide blood levels never exceeded 9.6 mg./100 cc. Thus it would appear that either the sulfanilamide is acetylated in the bovine mammary gland and passed to the milk in concentrations greater than those attained in the blood, or the acetylated form of the drug accumulates in the mammary gland and is excreted in the milk in greater than blood concentrations. These sulfanilamide permeability results on the cow correlate roughly with those reported for this drug in the human being.

DISCUSSION

The results recorded in these experiments tend to emphasize the probable significance of tissue permeability as a factor in the chemotherapeutic efficacy of a given drug. It would appear to be useless to expect five or six of the sulfonamides used here to be effective in curing infections involving the bovine mammary gland. Even assuming that

TABLE 2—Free Sulfonamide Concentrations in the Blood and in the milk after Oral Administration to Lactating Cows

Cow No.	Drug administered	Blood concentration mg./100 cc.					Milk concentration mg./100 cc.					
		6 hr.	10 hr.	22 hr.	34 hr.	46 hr.	Before	6 hr.	10 hr.	22 hr.	34 hr.	46 hr.
1	Sulfanilamide	4.8	6.2	5.9	2.4	1.0	0	3.6	5.0	5.7	2.8	1.0
2	Sulfanilamide	2.7	4.7	4.8	2.1	0.5	0	2.0	3.0	4.5	2.8	0.8
3	Sulfamerazine	5.6	6.4	10.2	5.2	2.0	0	1.8	1.5	2.6	1.5	0
4	Sulfamerazine	3.4	5.2	6.7	3.4	0	0	0	0	1.5	0.9	0
5	Sulfathiazole	2.4	1.4	1.4	0		0	0	0	0	0	
6	Sulfathiazole	0	1.6	1.8	0.8	0	0	0	0	0	0	0
7	Sulfapyrazine	1.5	1.3	2.9	1.0		0	0	0	0	0	0
8	Sulfapyrazine	0	1.4	1.7	0	0	0	0	0	0	0	0
9	Sulfapyridine	6.2	9.1	10.6	4.9	0.9	0	3.5	5.8	7.4	3.6	0
10	Sulfapyridine	3.8	6.2	7.2	3.3	0	0	2.0	3.8	5.8	3.0	0
11	Sulfapyridine	5.5	8.0	7.7	3.5	0	0	2.6	5.1	6.5	2.9	0
12	Sulfapyridine	4.5	6.2	8.4	5.1	1.7	0	3.2	4.1	6.4	3.8	1.4
13	Sulfadiazine	2.8	2.8	4.7	2.1		0	0	0	1.1	0	
14	Sulfadiazine	1.3	3.6	4.3	1.7	0	0	0	0	0	0	0
15	2-sulfanilamido-	6.7	7.5	13.4	12.3	6.3	0	0	0	0	0	0
16	5-bromopyrimidine	3.8	4.8	6.3	3.4	0	0	0	0	0	0	0
17	2-sulfanilamido-		3.1	8.4	5.6	1.6	0	0	0	0	0	0
18	5-chloropyrimidine	2.3	4.3	5.8	4.6	2.0	0	0	0	0	0	0

all other infected tissues were permeated by the drugs in therapeutic concentrations, the mammary gland infections would persist and could serve as a focus for relapse after treatment. An analogous situation has been established by Schuhardt and Hemphill[5] in the case of penicillin therapy in experimental relapsing fever. Here, the meningovascular barrier prevented cure of the brain involvement which in turn resulted in relapse after treatment. Of course, it must be recognized that these mammary gland permeability experiments were done on supposedly normal cows. Pathologic tissues may show different permeability phenomena.

On the basis of these results, it would appear that sulfapyridine, when fed, might be expected to give the best therapeutic effect of any of the eight drugs tested, if the infection involved the mammary gland of the cow, and the permeability of the infected gland was comparable to that of the normal gland. If other involved tissues should show different permeability reactions, multiple or sequential sulfonamide therapy might prove more effective than single drug therapy.

TABLE 3—Comparison of Free and Total Sulfonamide Concentrations in Blood and in Milk after Oral Administration to Lactating Cows

Cow No.	Drug administered	Blood concentration mg./100 cc. 22 hr. F.	22 hr. T.	34 hr. F.	34 hr. T.	46 hr. F.	46 hr. T.	Milk concentration mg./100 cc. 22 hr. F.	22 hr. T.	34 hr. F.	34 hr. T.	46 hr. F.	46 hr. T.
1	Sulfanilamide	5.9	9.6	2.5	4.4	1.0	1.9	5.7	13.8	2.8	11.1	1.0	5.0
2	Sulfanilamide	4.8	7.6	2.1	4.9	0.5	0.8	4.5	17.6	2.8	13.8	0.8	3.9
3	Sulfamerazine	10.2	11.9	5.2	5.3	1.8	2.0	2.6	4.0	1.5	1.7	0	0
4	Sulfamerazine	6.7	7.0	3.4	3.4	0	0	1.5	2.7	0.9	1.8	0	0
5	Sulfathiazole	1.4	2.5	0	0			0	1.0	0	0		
6	Sulfathiazole	1.8	1.8	0.8	0.8	0		0	1.1	0	1.3	0	0
7	Sulfapyrazine	2.9	4.3	1.0	1.4			0	2.3	0	0		
8	Sulfapyrazine	1.7	1.7	0	0	0	0	0	0.7	0	0	0	0
9	Sulfapyridine	10.6	13.0	4.9	5.8	0.7	0.9	7.4	10.4	3.6	4.3	0	0.7
10	Sulfapyridine	7.2	7.8	3.3	3.8	0	0	5.8	7.2	3.0	3.9	0	0
11	Sulfapyridine	7.7	7.8	3.5	3.9	0	0	6.5	8.0	2.9	4.2	0	0
12	Sulfapyridine	8.4	8.7	5.1	5.1	1.6	1.7	6.4	7.1	3.8	4.6	1.4	1.4
13	Sulfadiazine	4.7	6.8	2.1	2.2			1.1	2.1	0	0		
14	Sulfadiazine	4.1	4.1	1.7	1.9	0	0	0	0	0	0	0	0
15	2-sulfanilamido-	13.4	16.1	12.3	14.4	6.3	6.7	0	4.2	0	0	0	0
16	5-bromopyrimidine	6.3	6.9	3.4	3.4	0	0	0	0	0	0	0	0
17	2-sulfanilamido-	8.4	11.6	5.6	6.3	1.7	1.8	0	3.5	0	0	0	0
18	5-chloropyrimidine	5.8	6.6	4.6	4.6	1.6	2.0	0	0.8	0	0	0	0

F.=free.
T.=total.

SUMMARY

1) Only sulfanilamide and sulfapyridine of eight sulfonamides fed came through the bovine mammary gland into the milk in concentrations of the free drug approaching those attained in the blood stream. Of the two, sulfapyridine reached higher concentrations both in the blood and in the milk.

2) All of the sulfonamides were acetylated to some extent, and some of each of the acetylated drugs appeared in the milk even when the free drug failed to be demonstrable therein. Acetylated sulfanilamide seemed to accumulate in the milk to a noticeable degree.

References

[1]Adair, F. L., Hesseltine, H. C., and Hac, L. R.: An Experimental Study of the Behavior of Sulfanilamide. J. Am. M. A., *111*, (1938): 766-769.

[2]Bauer, H., and Gunderson, M. F.: Para-aminosulfamidobenzene in Amniotic Fluid and Placental Blood. J. A. V. M. A., *93*, (1938): 118.

[3]Bratton, A. C., and Marshall, E. K., Jr.: A New Coupling Compound for Sulfanilamide Determination. J. Biol. Chem., *128*. (1939): 537-550.

[4]Pinto, S. S.: Excretion of Sulfanilamide and Acetylsulfanilamide in Human Milk. J. Am. M. A., *111*, (1938): 1914-1916.

[5]Schuhardt, V. T., and Hemphill, Emmarie C.: Brain Involvement as a Possible Cause of Relapse After Treatment in Spirochetal Relapsing Fever. Science, *103*, (1946): 422-423.

[6]Stewart, H. L., Jr., and Pratt, J. P.: Sulfanilamide Excretion in Human Breast Milk. J. Am. M. A., *111*, (1938): 1456-1458.

Listerellosis—Report of an Outbreak in Wyoming Sheep and Preliminary, Experimental Therapy in Rabbits

J. F. RYFF, D.V.M., AND A. M. LEE, D.V.M., M.S.
Laramie, Wyoming

If, as has been predicted, listerellosis may someday assume the importance of brucellosis as a public health problem, attention should be focussed on each new case until more is known of the disease.

This outbreak reported may really have begun in cattle, for, in the spring of 1944, in a feedlot with about 50 head of cattle, circling became very pronounced in 1 animal. The animal was killed, but gross and microscopic study of various organs, including the brain, was without significance. Cultural studies and animal inoculations were also negative. Two months later another steer was destroyed because of circling, and a small, gram-positive rod was recovered from the brain. By the time cultural studies and animal inoculation tests had revealed the organism to be a bacillus other than *Listerella monocytogenes*, the original material had been discarded. Microscopically, lesions suggestive of listerellosis were found in the brain stem (fig. 1 and 2).

In April, 1945, this same feeder met with trouble in lambs. All through the winter, these lambs had done well until about three weeks after corn ensilage was supplied as part of the ration. Within a week, 9 of the 240 had died, all of them showing the same symptoms of going off feed, circling, and then dying within twenty-four to seventy-four hours. Losses became so high that the flock was sold

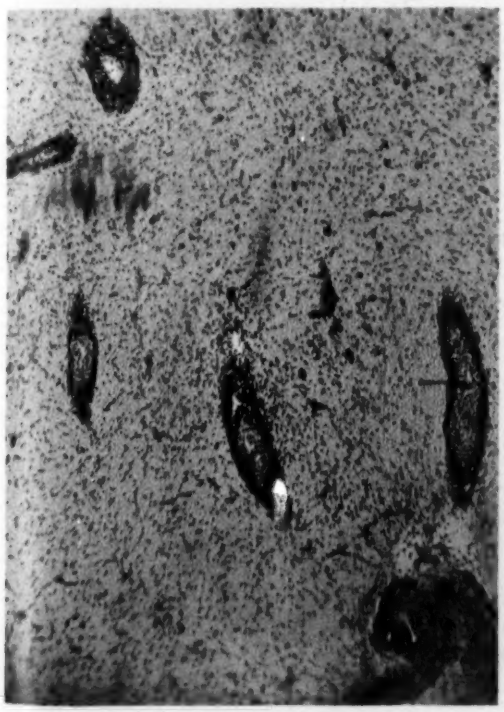

Fig. 1—Extensive perivascular cuffing in brain. Suspected listerellosis in steer. X65.

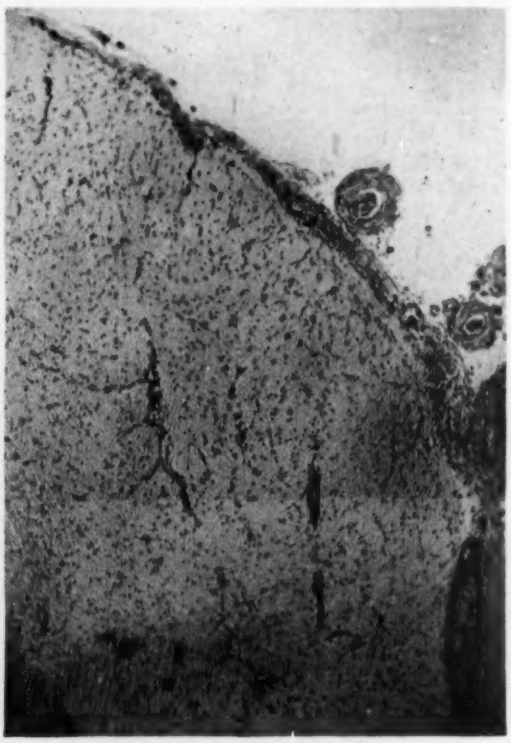

Fig. 2—Infiltration of meninges and thrombus of vessel. Suspected listerellosis in steer. X65.

Now with the Wyoming State Veterinary Laboratory (Ryff); Agricultural Experiment Station, University of Wyoming (Lee).

Approved for publication by J. A. Hill, director of the Wyoming Agricultural Experiment Station, Laramie.

Our appreciation is due Dr. J. B. Fuller who furnished the field material and history.

TABLE I—Young Rabbits Inoculated with 3 m.l.d. Listerella Monocytogenes Culture Jan. 18, 1946, 9 a. m.

Treatment	When treated		Died (date)	Died (time)
1. 10 U. streptomycin/Gm. intramuscularly	1-19-46	9 a. m. 12 noon 3 p. m.	1-20-46	11 a. m.
2. 40 U. streptomycin/Gm. intraperitoneally	1-19-46	9 a. m.	1-20-46	11 a. m.
3. 5 U. streptomycin/Gm. intramuscularly	1-19-46	9 a. m. 12 noon 3 p. m.	1-20-46	11 a. m.
4. 85 U. penicillin/lb. initially followed by 65 U. intramuscularly	1-19-46	9 a. m. 12 noon 3 p. m. 6 p. m.	1-20-46	8 p. m.
5. 125 U. penicillin/lb. intramuscularly	1-19-46	9 a. m. 12 noon 3 p. m.	1-20-46	11 a. m.
6. 625 U. penicillin/lb. intramuscularly	1-19-46	9 a. m. 12 noon 3 p. m.	1-20-46	8 p. m.
7. .025 gm. sodium sulfamerazine/lb. intravenously	1-19-46	9 a. m. 6 p. m.	1-20-46	11 a. m.
8. .025 gr. sodium sulfamerazine/lb. intravenously	1-19-46	9 a. m. 6 p. m.	1-20-46	11 a. m.
9. 1 gr. sodium sulfathiazole/lb. intravenously	1-19-46	9 a. m.	1-20-46	11 a. m.
10. 1 gr. sodium sulfathiazole/lb. intravenously	1-19-46 1-20-46	9 a. m. 6 p. m. 9 a. m. 8 p. m.	1-21-46	9 a. m.
11. 1 gr. sodium sulfapyridine/lb. intravenously	1-19-46 1-20-46 1-21-46 1-22-46	9 a. m. 6 p.m. 9 a. m. 8 p. m. 9 a. m. 9 a. m.	1-23-46	6 p. m.
12. 1 cc. disulfalac/lb. intravenously	1-19-46 1-20-46 1-21-46	9 a. m. 6 p. m. 9 a. m. 8 p. m. 9 a. m.	1-22-46	11 a. m.
13. 1 cc. disulfalac/lb. intravenously	1-19-46	9 a. m.	1-20-46	11 a. m.
14. 1 gr. SRDI/lb. intravenously	1-19-46	9 a. m.	immediately.	
15. 1 gr. SRDI/lb. intramuscularly	1-19-46	9 a. m. 6 p. m.	1-20-46	11 a. m.
16. Untreated			1-20-46	11 a. m.

Streptomycin supplied by Merck and Company. Sodium sulfamerazine supplied by Sharp and Dohme.

Disulfalac supplied by Pitman-Moore Company (2.5% sodium sulfathiazole, 2.5% sodium sulfapyridine, and 5.% dextrose in 1/6 M. sodium lactate).

SRDI supplied by W. B. Research Laboratory (described as sulph-recin-diamide-iodi type of sulfa preparation).

TABLE 2—Mature Rabbits Inoculated Intravenously with Listerella Monocytogenes and Treated with Sodium Sulfapyridine Intravenously

	Culture, Mar. 5, '47 1 p.m.	Sodium sulfapyridine	When treated	Disposal*	Gross lesions	Organism recovered†
A-1	0.5 cc.	Control		D—Mar. 8 a.m.	+	H,L,S
A-2	0.5 cc.	1 gr./lb.	Mar. 5, 5:30 p.m. Mar. 6, 8:30 a.m., 5:30 p.m. Mar. 7, 8:30 a.m., 5:30 p.m. Mar. 8, 8:30 a.m.	D—Mar. 15 a.m.	—	H,L,S
A-3	0.5 cc.	1 gr./lb.	Mar. 5, 5:30 p.m. Mar. 6, 8:30 a.m., 5:30 p.m. Mar. 7, 8:30 a.m., 5:30 p.m. Mar. 8, 8:30 a.m.	K—Mar. 15	+	H,L,S
A-4	0.5 cc.	1 gr./lb.	Mar. 5, 5:30 p.m. Mar. 6, 8:30 a.m., 5:30 p.m. Mar. 7, 8:30 a.m., 5:30 p.m. Mar. 8, 8:30 a.m.	K—Mar. 15	—	L,S
A-5	0.5 cc.	2 gr./lb.	Mar. 5, 5:30 p.m. Mar. 6, 8:30 a.m., 5:30 p.m. Mar. 7, 8:30 a.m., 5:30 p.m. Mar. 8, 8:30 a.m.	K—Mar. 15	—	H,L,S
A-6	0.5 cc.	2 gr./lb.	Mar. 5, 5:30 p.m. Mar. 6, 8:30 a.m., 5:30 p.m. Mar. 7, 8:30 a.m., 5:30 p.m. Mar. 8, 8:30 a.m.	K—Mar. 15	—	—
A-7	0.5 cc.	2 gr./lb.	Mar. 5, 5:30 p.m.	K—Mar. 27	—	—
A-8	0.25cc.	Control		K—Mar. 27	—	—

*D=died; K=killed.

†Heart, liver, and spleen cultured only.

for slaughter before feeding was completed. One of the lambs was submitted for diagnostic purposes. When received, its orientation was disturbed but no more than could be blamed to the slight wool blindness present. Taken at various times, its temperature was never excessively high (102.8 to 103.9 F.). In a week, incoördination had become progressively worse, and the lamb was killed. Except for a small abscess at the apex of the heart, no gross lesions were observed. Microscopically, the changes described in listerellosis were seen in the brain (fig. 3 and 4). From the brain stem was recovered a gram-positive, slightly pleomorphic rod with the cultural characteristics of *L. monocytogenes*. This organism cross-agglutinated with serums prepared against known cultures of *L. monocytogenes*,* on intravenous inoculation, produced the typical pathologic changes described for rabbits, and resulted in conjunctivitis and keratitis on conjunctival sac inoculation of rabbits, as noted by Julianelle and Pons,[1] Julianelle,[2,3] and Julianelle and Moore.[4] From time to time since this isolation, we have met with cases in cattle that were highly suggestive of listerellosis, but no material has been available for laboratory confirmation. In view of this, and the marked tenacity of the infection at the ranch mentioned, an attempt was later made to evaluate various therapeutic agents.

EXPERIMENTAL

On resuming experimentation, approximate-

*Secured from Dr. Robert Graham, University of Illinois. Urbana.

TABLE 3—Mature Rabbits Inoculated Intravenously with 0.5 cc. Listerella Monocytogenes Culture March 12, 1947, 3 p. m. and Treated Intravenously with Penicillin

	Amt. (U./lb.)	Treated	Disposal*	Gross lesions	Organism Recovered†
A-9	Control	All treated Mar. 12, 7:00 p.m. and Mar. 13, 8:30 a.m.	D—Mar. 18 a.m.	+	L,S
A-10	1,000		K—Mar. 27	—	—
A-11	1,000		D—Mar. 18 a.m.	+	H,L,S
A-12	2,000		K—Mar. 27	—	—
A-13	2,000		K—Mar. 27	±	—
A-14	3,000		D—Mar. 18 p.m.	+	L,S
A-15	3,000		K—Mar. 27	±	H,L,S

*D=died; K=killed.

†Heart, liver, and spleen only cultured.

ly a year after the first series of trials, the original culture isolated from sheep, although uniformly fatal to young rabbits originally in 0.25-cc. amounts, did not kill in amounts of

TABLE 4 — Mature Rabbits Inoculated Intravenously with 0.5 cc. Listerella Monocytogenes Culture March 27, 1947, Plus 20 cc. of Rabbit Antiserum

Source	Agglutination titer against challenge strain	Died (date)	Gross lesions	Organism recovered†
A-16 homologous	1:400	Mar. 31	+	H,L,S
A-17 ovine orig.*	1:100	Mar. 31	+	H,L,S
A-18 ovine orig.*	1:200	Mar. 31	+	H,L,S

*Cultures supplied by Dr. Graham.

†Heart, liver, and spleen only cultured.

10, 15, and 25 cc. After five intracerebral and two intravenous passages in rabbits, the virulence was again restored so that 0.5 cc. of culture was regularly fatal on intravenous inoculation. With this reminder that a supposedly avirulent culture still had virulent possibilities, no immunization attempts were made with the avirulent culture.

Table 1 gives the results secured with various agents when these were given twenty-four hours after the intravenous inoculation of three times the minimum lethal dose (m.l.d.) of culture. In view of the heavy exposure, these results should be interpreted on prolongation of survival time. On this basis, sodium sulfapyridine, disulfalac, sodium sulfathiazole, and penicillin might be thought to have some value in treatment.

Later, some of these agents were used more extensively. In table 2, the rabbits were exposed to approximately one m.l.d. of culture, and treatment with sodium sulfapyridine was begun four hours later. Of 6 rabbits so handled, 5 might be considered "cured," and, in 5, gross lesions of infection were not apparent on autopsy. However, in only 2 of the 6 rabbits could the organism be considered eliminated. In table 3, under the same conditions, penicillin, given only twice, however, might be considered responsible for 4 "cures." While the incidence of lesions attributed to *L. monocytogenes* is greater in this group than the sulfapyridine-treated rabbits, in only half of the animals could the organism be recovered on autopsy. Table 4 illustrates the fail-

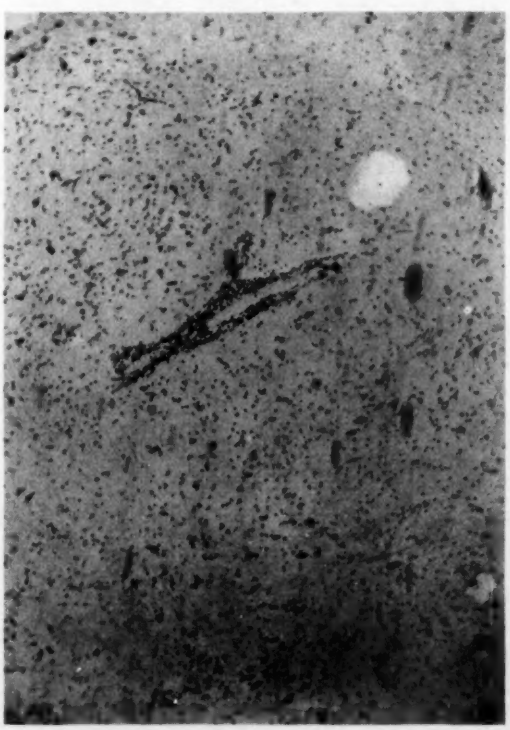

Fig. 3—Perivascular infiltration of brain. Listerellosis in sheep. X65.

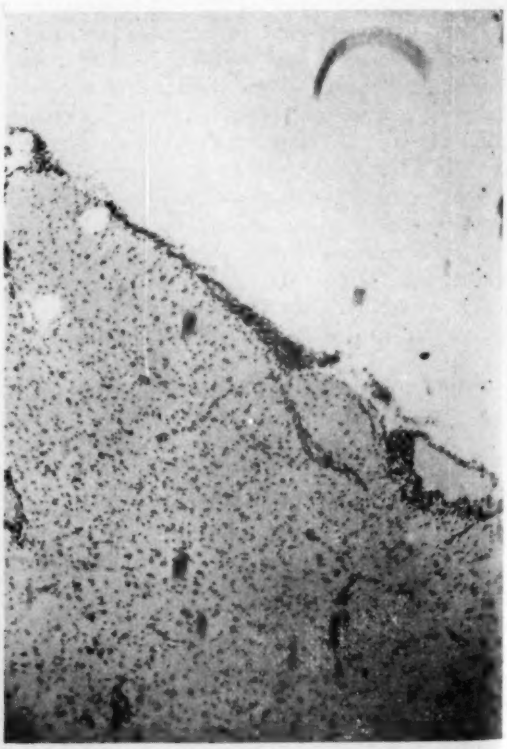

Fig. 4—Meningeal infiltration. Listerellosis in sheep. X65.

ure of antiserum to protect against the infection, even when given simultaneously with the inoculant culture.

SUMMARY

Several cases of suspected listerellosis in steers occurred on a Wyoming ranch and were followed by an outbreak in sheep on the same premises, which was confirmed as listerellosis by the laboratory. Of a number of therapeutic agents used on experimentally infected rabbits, sodium sulfapyridine and penicillin gave the most promising results.

References

[1]Julianelle, L. A., and Pons, G. A.: Identification of *Listerella monocytogenes*. Proc. Soc. Exptl. Biol. and Med., *40*, (1939) 362-363.

[2]Julianelle, L. A.: The Function of Listerella in Infection. Ann. Int. Med., *14*, (1940): 608-620.

[3]Julianelle, L. A.: Biological and Immunological Studies of Listerella. J. Bact., *42*, (1941): 367-383.

[4]Julianelle, L. A., and Moore, Elizabeth: Pathological changes in Listerella Infection, Particularly of the Eye. Am. J. Path., *18*, (1942): 813-825.

The Sensitivity of Bacteria of Animal Origin to Streptomycin

E. H. COLES, JR., D.V.M., M.S.

Ames, Iowa

STREPTOMYCIN, the antibiotic which was first described by Schatz, Bugie, and Waksman,[1] has been shown to be relatively active against various types of bacteria pathogenic to man.

Before this antibiotic may be used rationally for the treatment of disease conditions in animals, the sensitivity of the microörganisms pathogenic to animals must be determined. This study was undertaken to determine the effectiveness of streptomycin *in vitro* and *in vivo* against such bacteria.

EXPERIMENTAL METHODS

The streptomycin* was diluted in sterile physiologic salt solution and then diluted in tryptose blood agar in order to obtain the desired concentrations of 10.0, 5.0, 2.5, 1.0, 0.5, and 0.25 μg. (1 μg. is equal to 1 unit) per cubic centimeter. Controls in which no streptomycin was incorporated were used in all laboratory experiments.

The organisms to be tested were washed from a 24-hour tryptose agar slant culture and diluted to a concentration of approximately 3 million bacteria per cubic centimeter. These suspensions were streaked on the plates containing streptomycin and incubated for forty-eight hours at 37 C. At the end of this period, the plates were removed from the incubator and the results recorded as + for abundant growth equal to that of the control in which no streptomycin was incorporated, ± for partial inhibition, and − for complete inhibition with no visible colonies. The cultures tested were, for the most part, stock cultures.

In order to determine the bactericidal effect of streptomycin and to compare it with the bacteriostatic effect, the rotating rack technique developed by Lockwood[2] and used for penicillin determinations by Robinson[3] was adopted. The rack, a conical metal disc, was adjusted so that it rotated vertically, completing one revolution each minute. In this manner a complete and continuous mixing of the broth was obtained.

In these determinations, one drop of a six-hour broth culture of the organism to be tested was placed in tryptose broth tubes containing the different concentrations of streptomycin in 2 cc. of broth. A plate count of the broth in these tubes was made immediately before they were placed on the rotating rack. The medium used in this and all subsequent counts was tryptose agar.

These tubes, after inoculation, were placed on the rack in an incubator at 37 C. The rack was stopped every two hours during the course of the experiment and 0.1 cc. of the broth was removed aseptically, diluted, and plated. All of these plates were then incubated for forty-eight hours and the number of colonies counted. The counts were recorded as being indicative of the number of viable bacteria present in the broth at the time the sample was taken.

Some of the more susceptible strains of *Pasteurella multocida* were tested *in vivo* using the white mouse as the test animal. The mice were inoculated intraperitoneally with 0.1 cc. of an undiluted eight-hour culture of the organism tested. Treatment with streptomycin was begun an hour after inoculation and continued at varying intervals. All doses of the drug were administered intraperitoneally.

Cultures were made from the heart blood and liver in order to determine the cause of death.

RESULTS

Agar Plate Method.—The activity of streptomycin against several microörganisms as determined by the agar plate method is shown in table 1. The end point of sensitivity as recorded in this table was considered as the plate on which no colonies were visible.

Of the bacteria studied, *Aerobacter aerogenes, Bacillus anthracis, Brucella abortus, Brucella melitensis, Brucella suis, Corynebacterium pyogenes, Listerella monocytogenes, Pasteurella hemolytica, P. multocida,* and *Proteus ammoniae* were the most susceptible to the action of streptomycin. The salmonellas,

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From the Department of Veterinary Hygiene, Iowa State College, Ames.

The writer wishes to thank Dr. I. A. Merchant for the aid and advice so kindly given in the course of this work and in the preparation of this manuscript. He is also indebted to Dr. R. A. Packer for many suggestions offered and the cultures that were supplied.

*The streptomycin was furnished by Merck & Co., Inc.

streptococci, *Erysipelothrix rhusiopathiae*, and *Pseudomonas aeruginosa* showed relatively greater resistance to the action of this antibiotic.

Although *P. multocida* is relatively susceptible to the action of streptomycin, the data given in table 2 emphasize the strain variability that exists.

Of the 78 strains of *P. multocida* that were tested, 33 (41.8%) were either completely or partially inhibited in a concentration of 1.0 μg. of streptomycin/cc. of agar. Thirty-five strains (44.3%) were inhibited by 2.5 μg/cc. Three strains were inhibited by a concentration of 0.5 μg./cc. Two strains were completely resistant to 5 μg./cc. but partially inhibited by a concentration of 10.0 μg./cc.

The source of the strain, date of isolation, and the animal from which it was isolated seemed to have little effect upon the sensitivity of these organisms. Other workers have reported the efficacy of streptomycin in the treatment of tularemia in man. [5, 6, 7, 8, 9] Santivanez [4] reported a strain of *P. avicida* that was completely inhibited in a concentration of 6.25 μg./cc. in twenty hours.

With the exception of the staphylococci, the source and date of isolation seemed to have little effect upon the sensitivity of the strains tested. In general, it was found that the strains of *Staphylococcus aureus* isolated from dogs were more susceptible to the action of streptomycin than strains isolated from other sources. Of the five canine strains tested, four were inhibited by 1 μg./cc. and one culture showed no growth in a concentration of 0.5 μg./cc.

Rotating Rack Technique.—The effect of streptomycin upon the various organisms having been determined by the agar plate method, the bactericidal and bacteriostatic effects were determined by use of the rotating rack technique.

The bacteriostatic effect of streptomycin upon the test strain of *Escherichia coli* is shown in table 3.

It will be noted, that in none of the concentrations was the broth completely sterilized, but in both the 5- and 2.5-μg. concentrations there was some inhibition. A concentration of 5 μg. demonstrated a definite bacteriostatic effect upon the organism.

In other tests of the same type, it was found that a concentration of 20 μg./cc. of broth sterilized the culture in six hours. In all agar plate determinations, the organism was inhibited by a concentration of 2.5 μg. of streptomycin/cc.

The strain of *P. multocida* reported in table 4 was isolated from the wattle of a chicken and was completely inhibited by a concentration of 2.5 μg./cc. on the agar plate and partially inhibited by 1 μg. per cc. Two and ½ μg. of streptomycin/cc. of broth completely sterilized the culture at the end of a six-hour period. Although a concentration of 1.0 μg./cc. demonstrated a very definite bacteriostatic effect on this organism, at the end of twenty-four hours the culture was multiplying rapidly.

The salmonellas were found to be resistant to the action of streptomycin on the agar plate, and the same was found to be true when the rotating rack technique was used. A strain of *Salmonella typhisuis* which was partially inhibited by a concentration of 10 μg. of streptomycin/cc. on the agar plate was tested on the rotating rack. The results are recorded in table 5. A concentration of 20 μg. of streptomycin/cc. of broth sterilized the cul-

TABLE I—Range in Sensitivity of Bacteria of Animal Origin to Streptomycin as Determined by the Agar Plate Method

Organism	No. strains tester	Range in sensitivity (μg./cc.)
A. aerogenes	1	2.5
B. anthracis	4	0.25– 1.0
Br. abortus	17	1.0 – 2.5
Br. melitensis	1	1.0
Br. suis	3	1.0 – 2.5
C. pseudotuberculosis	1	1.0
C. pyogenes	13	0.25– 10.0
C. renalis	3	0.5 – 2.5
Ery. rhusiopathiae	1	10.0
Esch. coli (Test Strain)	1	2.5
K. pneumoniae	1	5.0
L. monocytogenes	1	1.0
P. hemolytica	3	0.5 – 2.5
P. multocida	78	1.0 – 10.0
Proteus ammoniae	1	1.0
Pseudomonas aeruginosa	1	10.0
S. choleraesuis	11	5.0 – 10.0
S. enteritidis	1	5.0
S. paratyphi C.	2	10.0 –>10.0
S. pullorum	9	5.0 – 10.0
S. typhimurium	3	10.0
S. typhisuis	1	10.0
Str. aureus	42	0.5 – 10.0
Str. agalactiae	2	10.0
Str. canis	1	10.0
Str. dysgalactiae	1	5.0
Str. gallinarum	1	10.0
Str. uberis	1	10.0
Str. zoöepidemicus	1	10.0

ture in four hours, while a concentration of 5 μg. exhibited only a slight suppressing action.

TABLE 2—Sensitivity of Strains of Pasteurella Multocida to Streptomycin as Measured by the Agar Plate Method

Number of cultures	Streptomycin μg./cc. of medium					
	10.0	5	2.5	1.0	0.5	0.25
2	±	+	+	+	+	+
2	—	±	+	+	+	+
3	—	—	+	+	+	+
8	—	—	±	+	+	+
27	—	—	—	+	+	+
29	—	—	—	±	+	+
4	—	—	—	—	+	+
3	—	—	—	—	±	+

IN VIVO EXPERIMENTS

Although the amount of streptomycin available was very limited, a few experiments were conducted using the white mouse as the experimental animal.

Twenty-five mice were infected with 0.1 cc. of an undiluted eight-hour culture of *P. multocida*, and one hour later streptomycin was administered intraperitoneally. Five of the mice were given a single dose of 1,000 μg.; of those mice, 3 were dead at the end of four days, and the other 2 were living at the end of seven days.

A single dose of 500 μg. slowed the rate of death but did not save the lives of any mice. Single doses of 100, 50, and 25 μg. had no effect upon the rate of death.

It was obvious from this experiment that single doses of the antibiotic would not be sufficient to preserve the lives of any test animals. The results of an experiment in which the varying amounts of streptomycin were administered at one hour, six hours, eighteen hours, and twenty-four hours after infection are recorded in table 6.

The mice that received a total dosage of 4,000 and 2,000 μg. of streptomycin survived the infection. Those mice that received a total of 1,000 μg. over a twenty-four hour period did not all survive the infection, but the rate of death was much slower than that of the untreated mice.

TABLE 3—Effect of Streptomycin upon Escherichia Coli (Test Strain), Rotating Rack Technique

Streptomycin μg./cc. of broth	No. of viable bacteria/0.1 cc. of broth					
	Time in hours					
	0	2	4	6	8	10
5.0	2,500,000	56,000	2,400	3,400	400	11,000
2.5	3,500,000	6,000,000	59,000	69,000	12,300	**
1.0	3,000,000	**	**	**	**	**
0.5	2,750,000	**	**	**	**	**
None	3,000,000	**	**	**	**	**

**Too numerous to count.

TABLE 4—Effect of Streptomycin upon Pasteurella Multocida, Rotating Rack Technique

Streptomycin μg./cc. of broth	No. of viable bacteria/0.1 cc. of broth					
	Time in hours					
	0	2	4	6	8	24
5.0	3,000,000	1,200	0	0	0	0
2.5	2,500,000	410,000	100	0	0	0
1.0	3,000,000	1,500,000	490,000	800,000	330	**
0.5	3,750,000	2,000,000	1,200,000	2,000,000	**	**
None	3,000,000	**	**	**	**	**

**Too numerous to count.

The strain of *P. multocida* used in the preceding experiments was 41305, the U.S.BAI antiserum test strain. This organism was found to be inhibited by 1 μg./cc. when tested by the agar plate method.

Another strain of *P. multocida* (PM102), isolated from a porcine spleen, was found to be inhibited by a concentration of 2.5 μg. of the drug /cc. *in vitro*. This organism killed mice in twenty-four hours when 0.1 cc. of an undiluted eight-hour culture was administered intraperitoneally.

It was found that a total dosage of 4,000 μg. administered over a 24-hour period was not sufficient to preserve the life of all mice treated. However, a slight effect of the drug was noticed in the mice which received the higher dosages of streptomycin in that these mice did not die as rapidly as did the untreated animals.

DISCUSSION

In the course of this study, it has been demonstrated that the organisms examined show a wide range of susceptibility to streptomycin.

The organisms found to be most sensitive to streptomycin include *B. anthracis*, *Br. abortus*, *Br. suis*, *Br. melitensis*, *P. hemolytica*, *P. multocida*, *Corynebacterium renalis*, *C.*

pyogenes, *Sta. aureus*, *Proteus ammoniae*, *L. monocytogenes*, and *A. aerogenes*. The streptococci, salmonellas, and a few strains of Corynebacterium were found to be relatively resistant to the action of this antibiotic.

TABLE 5—Effect of Streptomycin on Salmonella Typhisuis, Rotating Rack Technique

Streptomycin µg./cc. of broth	No. of viable bacteria/0.1 cc. of broth					
	Time in hours					
	0	2	4	6	8	10
20	300	300	0	0	0	0
5	700	900	1,000	50,000	7,000	**
1	300	9,000	61,000	150,000	900,000	**
0.25	4,000	300,000	91,000	360,000	600,000	**
None	800	12,000	150,000	800,000	**	**

**Too numerous to count.

It was noted particularly with *P. multocida* that a few strains of the organism were found to be inhibited by low concentrations of streptomycin, while others from the same source were found to be quite resistant to the drug. These results indicate that the sensitivity of a particular organism to streptomycin is more characteristic of the strain than of the species. This fact must be taken into consideration when this drug is employed as a therapeutic agent. The success of treatment with streptomycin will, in a large part, depend upon the strain of organism encountered, as well as the species.

This same type of strain variability was noted with *C. pyogenes* and *Sta. aureus*. As will be noted in table 1, the strains of *C. pyogenes* tested varied in susceptibility from 0.25 µg. to 10.0 µg./cc. when tested by the agar plate method. *Sta. aureus* strains tested varied in susceptibility from 0.5 µg. to 10.0 µg./cc.

The explanation of the phenomenon of strain variability is, at the present time, unknown. Although the pH of the medium, and the presence of buffers seem to have a deleterious effect upon the action of streptomycin, these factors are not significant here when the strain susceptibility is being discussed. The pH of all mediums used was adjusted to 7.2. No buffers other than those normally present in the blood were added to the medium.

The source of the culture and the date of isolation, with only one exception, seemed to have little, if any, effect upon the sensitivity of the various strains of organisms to streptomycin. Among the strains of *Sta. aureus* studied, it was found that those isolated from dogs showed more susceptibility to the action of streptomycin than did the staphylococci isolated from other sources.

In general, the gram-negative organisms showed more sensitivity to streptomycin than did the gram-positive organisms. The notable exceptions to this were *B. anthracis*, *Sta. aureus*, and *L. monocytogenes*. Three of the four cultures of *B. anthracis* tested showed extreme sensitivity to the drug, as they were inhibited by 0.25 µg/cc. This fact would warrant further study on this organism to determine the effectiveness of streptomycin *in vivo*.

The results obtained by the rotating rack technique paralleled the results obtained when the agar plate method of sensitivity determination was used. This can be noted in table 4. The strain of *P. multocida* used in this experiment was completely inhibited by 2.5 µg. when tested by the agar plate method

TABLE 6—Efficacy of Streptomycin in Mice Infected with Pasteurella multocida

Organism ..... *Pasteurella multocida*
Age of culture ..... 8 hours
Infection ..... 0.1 cc. of the undiluted culture intraperitoneally.
Treatment ..... Streptomycin administered intraperitoneally 1, 6, 18, and 24 hr. after infection.

No. of Mice	µg. per dose	Total dosage	Number of mice surviving in days						
			1	2	3	4	5	6	7
5	1,000	4,000	5	5	5	5	5	5	5
5	500	2,000	5	5	5	5	5	5	5
5	250	1,000	5	3	3	1	1	1	1
5	100	400	1	0	0	0	0	0	0
5	None	None	0	0	0	0	0	0	0

and, when tested by the rotating rack technique, it was found that the same concentration sterilized the broth in six hours. These parallel results seem to indicate that streptomycin is equally as efficient in broth as when incorporated in agar.

In many instances, the number of bacteria present in the broth of the rotating rack decreased in the first few hours of incubation, and then a definite increase in the number was noted when the next plate count was made. Whether this is due to the fact that the organisms overcame the action of streptomycin and reëntered the logarithmic growth phase, or that certain cells developed in that short period a tolerance and resistance to the drug is a problem that will require further study.

A total dosage of 2,000 μg. was sufficient to protect mice from the effects of *P. multocida* 41305; however, a dose of 4,000 μg. was not enough to protect mice when strain PM 102 was used. This again emphasizes the fact that strain specificity is just as important as the species of organism.

CONCLUSIONS

1. *Bacillus anthracis*, *Brucella abortus*, *Brucella suis*, *Brucella melitensis*, *Pasteurella hemolytica*, *Pasteurella multocida*, *Corynebacterium pyogenes*, *Staphylococcus aureus*, *Proteus ammoniae*, *Listerella monocytogenes*, and *Aerobacter aerogenes* are relatively sensitive to the action of streptomycin.

2) The salmonellas, streptococci, and some strains of Corynebacterium were found to be comparatively resistant to the action of streptomycin.

3) A large degree of variation of susceptibility of strains of organisms to this antibiotic exists.

4) The susceptibility of species to the action of streptomycin is not confined to the gram-negative group of bacteria.

5) The variation in sensitivity of various strains within a species is exhibited *in vivo* as well as *in vitro*.

6) The results obtained by the rotating rack technique paralleled the results obtained when the agar plate method of sensitivity determination was used.

References

[1]Schatz, A., Bugie, E., and Waksman, S. A.: Streptomycin, a Substance Exhibiting Activity Against Gram-negative and Gram-positive Bacteria. Proc. Soc. Exptl. Biol. Med., *55*, (1944): 66-69.

[2]Lockwood, J. S.: Studies on the Mechanism of the Action of Sulfanilamide. J. Immunol., *35*, (1938): 155-191.

[3]Robinson, H. J.: Toxicity and Efficacy of Penicillin. J. Pharmacol. and Exptl. Therap., *77*, (1943): 70-79.

[4]Santivanea, J.: Antibacterial Action of Streptomycin. Cornell Vet., *36*, (1946): 214.

[5]Heilman, F. R.: Streptomycin in the Treatment of Experimental Tularemia. Proc. Staff Meetings Mayo Clinic, *19*, (1946): 553-558.

[6]Peterson, R. L., and Parker, R. R.: Tularemic Pneumonia Treatment with Streptomycin. Pub. Health Rep., *61*, (1946): 1231-1234.

[7]Chapman, S. S., Coriell, L. L., Kowal, S. F., Nelson, W., and Downs, C. M.: Studies on Streptomycin, Therapy of Experimental Tularemia in White Mice. J. Bact., *51*, (1946): 607.

[8]Foshay, L., and Pasternack, A. B.: Streptomycin Treatment of Tularemia. J. Am. M. A., *120*, (1946): 393-398.

[9]Atwell, R. J., and Smith, D. T.: Primary Tularemia Pneumonia Treated with Streptomycin, a Report of Two Cases. South. M. J., *39*, (1946): 858-860.

Renal Tubular Excretion of Penicillin as Inhibited by Caronamide

KARL H. BEYER, Ph.D., M.D.
Glenolden, Pennsylvania

It is well known that the greatest limiting factor in the use of penicillin is its very rapid elimination from the blood stream. It has been shown both in man and in experimental animals that the rate of penicillin excretion is of the order of renal plasma flow.[1, 2] This could be taken to mean that at ordinary therapeutic blood levels of the drug it is completely removed from the plasma as it circulates through the kidneys. Therefore, it seems quite certain that the excretion of penicillin is by way of the renal tubules as well as the glomeruli. Actually, this tubular excretion of penicillin may account for some 80 per cent of its elimination by the kidney. This being the case, any adjuvant that would inhibit the tubular excretion of penicillin would result in as much as a fivefold increase in its physiologic economy.

In 1944, we reported that p-aminohippurate (PAH), a compound which is used to measure tubular excretory function and renal plasma flow, was capable of inhibiting the tubular excretion of penicillin when administered in large dosages.[3] The fact that this inhibition of penicillin excretion was accomplished by the injection of PAH indicated that these two compounds were excreted by a renal tubular mechanism common to both agents. The mode of action of PAH, then, was in competition with penicillin on a "mass action" basis for excretion by a renal transport mechanism common to both agents.

Subsequent clinical results substantiate the fact that the coadministration of PAH enhanced substantially the plasma concentration of penicillin.[4, 5, 6] However, there were two serious limiting factors in the use of PAH for this purpose. One was the tremendous dosages of PAH necessary, since that compound was excreted essentially as rapidly as penicillin, and the second that the PAH had to be administered by constant intravenous infusion.

However, encouraged by this measure of success, we attempted to find an agent that would accomplish this objective of inhibiting the tubular excretion of penicillin at a relatively low dosage when administered either orally or parenterally. It seemed possible to us that an agent could be found which was similar enough to penicillin or other compounds excreted by this mechanism to have an affinity for that system and yet not be

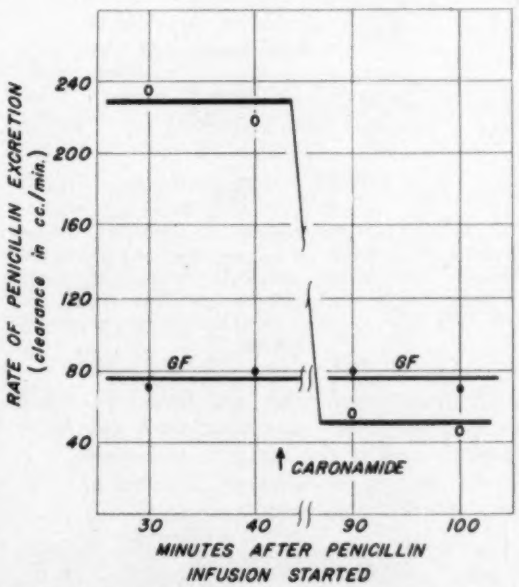

Fig. 1—The renal clearance of crystalline sodium penicillin G administered by venoclysis and glomerular filtration rate (GF) before and following the intravenous administration of sodium caronamide. The initial intravenous dose of caronamide (calculated as the acid) was 30 mg./kg. and this was followed by a venoclysis of the drug wherein the dosage was 25 mg./kg. of body weight administered at a rate of 3 cc./minute throughout the remainder of the experiment. The arrow indicates the time of the initial injection of caronamide.

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From the Department of Pharmacology, the Medical Research Division, Sharp & Dohme, Inc., Glenolden, Pa.

Tables 1 and 2 and figure 8 (redrawn) have been included with the kind permission of the authors, Drs. W. F. Verwey and A. K. Miller, and the editor of the Proceedings of the Society for Experimental Biology and Medicine.

excreted by the tubules. The mode of elimination of such an agent would be restricted essentially to glomerular filtration. It was desirable to have this agent well absorbed on oral administration and with a low order of toxicity. A compound was found which seemed to accomplish this purpose.[7]

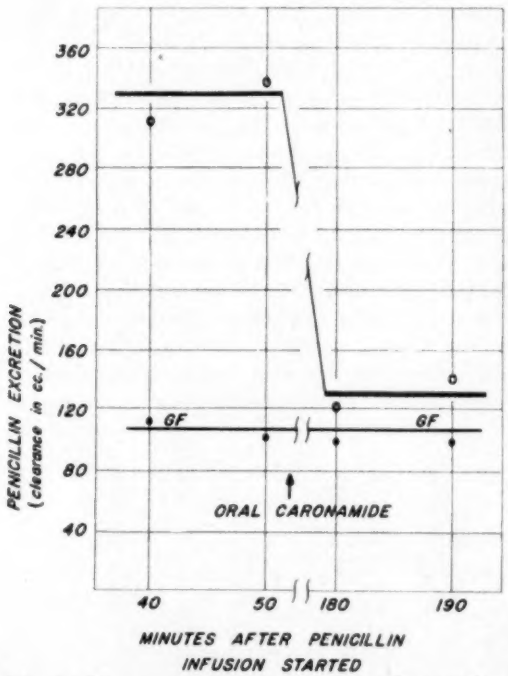
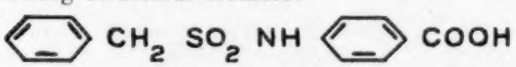


Fig. 2—The renal clearance of crystalline sodium penicillin G administered by venoclysis and glomerular filtration (GF) following a single oral dose of 50 mg. of caronamide/kg. of body weight. The arrow indicates the time at which the caronamide was administered.

The compound that was found to fulfill most closely the aforementioned requisites has been given the name "caronamide." It is also known as "staticin" and has the following structural formula:



The chemical name for this compound is 4'carboxyphenylmethanesulfonanilide. Its acid form is a relatively insoluble, colorless, crystalline material having no odor and little or no taste. As a mono or disodium salt it is readily soluble in water, and solutions of the agent can be prepared by dissolving the acid in dilute sodium hydroxide or sodium bicarbonate solution.

EXPERIMENTAL

The inhibitory effect of caronamide on the renal clearance of penicillin can be demonstrated by an experiment such as is represented in figure 1. It may be seen in the control phase of the experiments that the renal clearance of penicillin approximates the renal plasma flow for a dog of this size. In the second phase of the experiment, wherein caronamide was administered together with penicillin, the renal clearances of the antibiotic agent were depressed below the corresponding creatinine clearances. In the dog, creatinine clearance may be taken as a measure of the glomerular filtration rate. The decrease in penicillin clearance below the glomerular filtration rate is accounted for by the fact that a considerable amount of the penicillin is bound on plasma protein [8] and hence is not available for ultrafiltration. Corrected for binding, the clearance of penicillin following caronamide approximates the glomerular filtration rate.

Whereas amorphous commercial penicillin was used in the course of this experiment, it has been demonstrated that this inhibitory

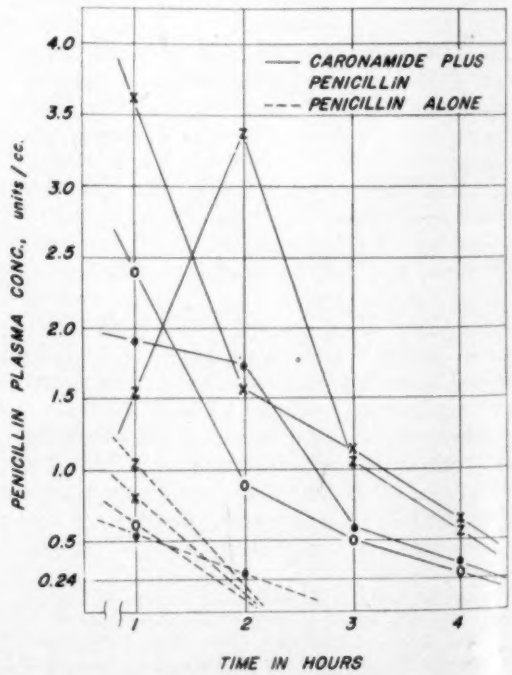


Fig. 3—The plasma concentration of penicillin following the oral administration of 100,000 units alone and with a 60 mg./kg. of body weight oral dose of caronamide administered simultaneously. The broken line represents the dosage response control curves for penicillin administered by itself. The solid line represents the penicillin curves when the antibiotic agent and the caronamide are administered together.

effect of caronamide was independent of the type of penicillin used. Beyer, Miller, Russo, Patch, and Verwey [9] reported that the normal renal clearance of penicillin F, G, K, and X approximated the renal plasma flow in the dog. Under the influence of caronamide, the clearance of all four compounds was decreased to, or below, the glomerular filtration rate.

It should be apparent that for widespread clinical usage such a compound as caronamide should be active on oral administration regardless of the mode of administration of penicillin itself. Figure 2 illustrates an experiment wherein penicillin was administered intravenously to dogs at a constant rate. The caronamide was administered orally as a single dose. One sees in this illustration that the orally administered dose of caronamide was capable of very materially decreasing the renal clearance of penicillin as evidenced by its absolute value and the clearance ratio. It has been found that the degree of the effect of caronamide is a function of its dosage to the point of complete inhibition of the tubular excretion of penicillin.

TABLE 1——Survival Percentages of Mice Infected with Type I Pneumococci and Receiving Penicillin with Caronamide and Penicillin Alone

Penicillin Dosage*	Penicillin with Caronamide**	Penicillin Alone
2 units	0	—
4 "	35	—
8 "	84	—
16 "	—	5
32 "	—	10
64 "	—	65
128 "	—	85
PD_{50}	5.0 units	55 units

*Doses given intramuscularly every 6 hours for nine doses

**10 mg given orally every 6 hours for nine doses

Although penicillin is not well absorbed from the gastrointestinal tract, it was thought that if caronamide and penicillin could be administered together orally the resulting antibiotic blood levels might be sufficiently enhanced to make this use of penicillin more rational. Accordingly, experiments were designed whereby the changing plasma concentration could be followed when 100,000 units of the agent were administered orally at four-hour intervals alone and with the coadministration of 60 mg./kg. of caronamide. Figure 3 summarizes one of these experiments. The broken-line curves are drawn through the plasma concentrations that followed the oral dosage of 100,000 units of penicillin alone. In only one instance was the antibiotic plasma concentration above the lower limits of our Florey cup-plate assay method (0.24 units/cc.) two hours following its ingestion. When the penicillin was administered with caronamide, the antibiotic plasma levels were strikingly higher than the previous control penicillin blood levels. Moreover, at the end of four hours all the antibiotic plasma levels were above the 0.24 unit/cc. limit of our assay.

In the evaluation of a new therapeutic agent such as caronamide, it is perhaps as important to determine its lack of effect on other kidney functions as to demonstrate its inhibitory effect on the tubular excretion of penicillin. Obviously, one gains little if, by suppressing the tubular excretion of penicillin, one also impairs the renal functions in general. To study any chronic toxicity the drug might produce, it was given to dogs for four weeks in divided oral dosages at four-hour intervals. The total daily dosage was 0.5, 1.0, and 1.5 Gm./kg./day to each of three groups of 12-month-old Beagle hounds. Initially, a control period was allowed for each dog wherein its normal blood and urine chemistry, hematology, weight, physical appearance, etc. were observed. Thereafter, the drug was administered and the studies continued for the duration of the experiment. At the termination of the trials, autopsies were performed on the dogs, and tissues were taken for microscopic examination.

Figures 4 through 7 summarize data on 1 control dog and a representative animal from the 1.0 Gm./kg./day group. It may be seen that the changing from a meat and pellet diet to an all pellet diet in the control phase of the experiment produced a greater effect on blood and urine chemistry than did caronamide. It could not be said that the administration of caronamide influenced the hematologic patterns of the dogs. Some of the dogs lost as much as 1 kg. during the experiment, but the change in weight was not related to dosage. The dogs were in excellent health at the time

TABLE 2—Survival Percentages of Mice Infected with Eberthella Typhosa and Receiving Penicillin with Caronamide and Penicillin Alone

Penicillin Dosage*	Penicillin with Caronamide**	Penicillin Alone
100	10.5	—
200	10	—
400	10	10.5
800	95	5.3
1600	—	30
3200	—	90
PD_{50}	515 units	1900 units

*Doses given intramuscularly every 6 hours for nine doses

**10 mg given orally every 6 hours for nine doses

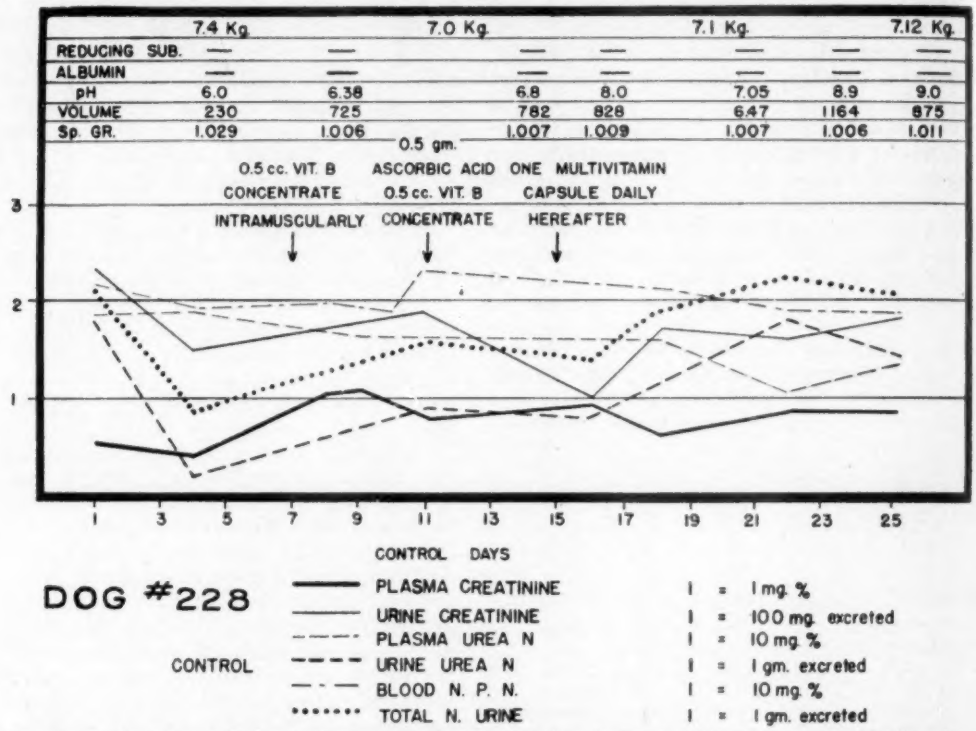

7.4 Kg.
7.0 Kg.
7.1 Kg.
7.12 Kg.
REDUCING SUB.
ALBUMIN
pH 6.0 6.38 6.8 8.0 7.05 8.9 9.0
VOLUME 230 725 782 828 647 1164 875
Sp. GR. 1.029 1.006 1.007 1.009 1.007 1.006 1.011
0.5 cc. VIT. B CONCENTRATE INTRAMUSCULARLY
0.5 gm. ASCORBIC ACID 0.5 cc. VIT. B CONCENTRATE
ONE MULTIVITAMIN CAPSULE DAILY HEREAFTER
3
2
1
1 3 5 7 9 11 13 15 17 19 21 23 25
CONTROL DAYS
DOG #228
CONTROL
PLASMA CREATININE 1 = 1 mg. %
URINE CREATININE 1 = 100 mg. excreted
PLASMA UREA N 1 = 10 mg. %
URINE UREA N 1 = 1 gm. excreted
BLOOD N. P. N. 1 = 10 mg. %
TOTAL N. URINE 1 = 1 gm. excreted

Fig. 4—Control hematologic studies on dog 228 during the chronic toxicity experiments. Erythrocyte counts are expressed in millions, leucocyte counts in thousands, hemoglobin in grams, and hematocrit, neutrophils, and lymphocytes in per cent.

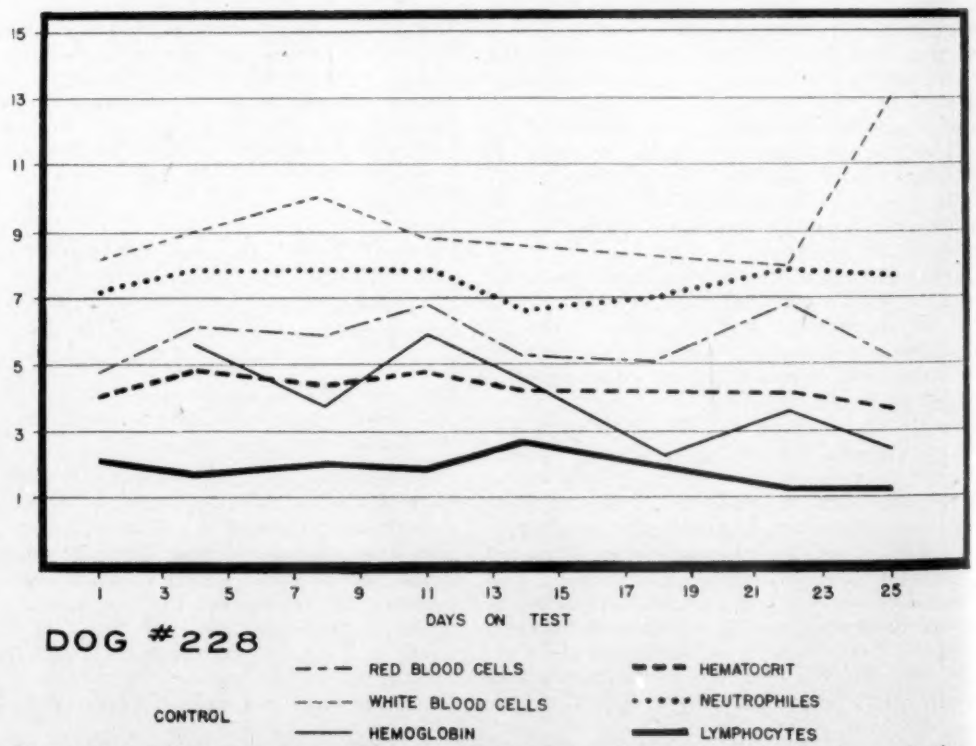


Fig. 5—Control blood and urine analyses on dog 228 during the toxicity study. The figures in the upper portion of the chart relate to body weight and urinalyses.

they were sacrificed. There was no gross or microscopic evidence of damage attributable to caronamide. The microscopic sections were studied by a consultant pathologist as well as by the author.

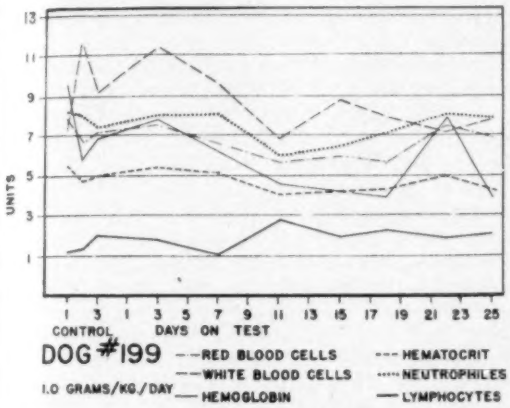


Fig. 6—The effect of 1.0 Gm. caronamide/kg. of body weight/day on the hematologic picture of dog 199. Erythrocyte counts are expressed in millions, leucocyte counts in thousands, hemoglobin in grams, and hematocrit, neutrophils, and lymphocytes in per cent.

The only noteworthy alteration in the metabolism of the dogs on the higher drug dosages was the presence of a nonfermentable, reducing substance in the urine. Although the reducing substance is not glucose, its identity is not established otherwise. A more detailed account of the toxicity studies on caronamide appears elsewhere.[10] Still other renal clearance measurements have been performed wherein it was found that, in dosages sufficient to halt the tubular excretion of penicillin, caronamide had no effect on the elimination of glucose, urea, amino acids, sulfonamides, or glomerular filtration. Also, it was found that similar dosages of the drug did not alter systemic blood pressure, respiration, or renal volume during its continued administration.[11]

The renal clearance of caronamide *per se* (not corrected for plasma binding) is about half the glomerular filtration rate.[11] It is possible that the binding of the agent on the plasma protein is at least 50 per cent, for it seems unlikely on a theoretic basis that caronamide could be reabsorbed by the tubules and yet inhibit such a tubular excretory mechanism as that for penicillin excretion. It is known that some caronamide is altered in the body and is excreted in this altered form. The na-

WEIGHT			9.4 Kg.		8.6 Kg.		8.8 Kg.		8.36 Kg.	
REDUCING SUB.	TRACE	TRACE	—	+	5 mg./cc.	6.67	4.1	3.3	3.12	5.0
ALBUMIN	TRACE	—	—	—	—	—	—	—	—	—
pH		6.4	8.25	7.3	7.12	7.48	7.4	8.3	8.9	8.9
VOLUME	117	305	80	1010	780	670	890	995	1015	925
Sp. GR.	1.043	1.038	1.021	1.010	1.012	1.012	1.011	1.014	1.013	1.014

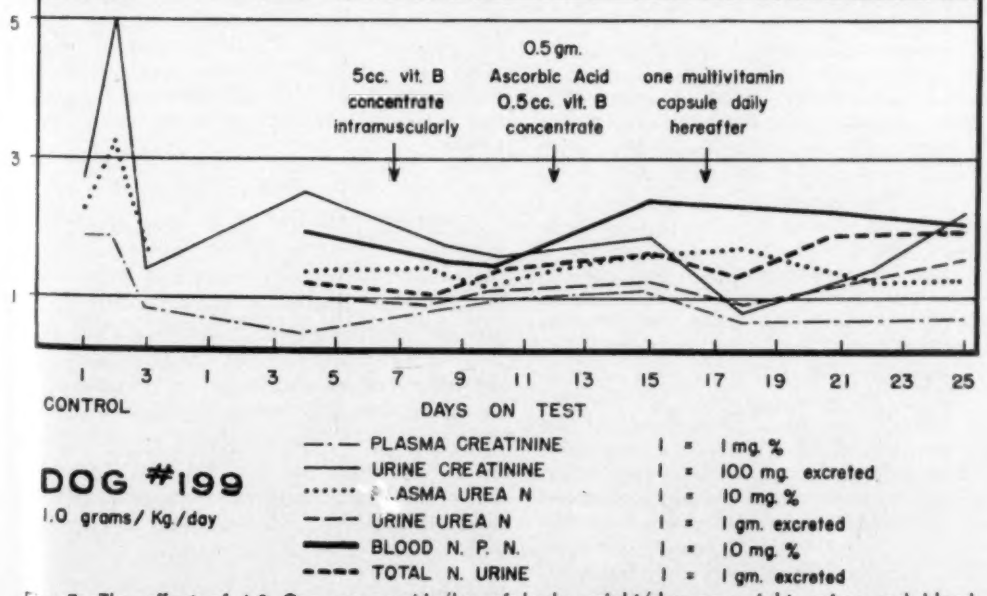


Fig. 7—The effect of 1.0 Gm. caronamide/kg. of body weight/day on weight, urine, and blood analyses of dog 199.

ture and the amount of this material have not been established.

Although it has been demonstrated that caronamide will increase the plasma concentration and the duration of the effect of individual dosages of penicillin, it is important to demonstrate that this results in the increased therapeutic usefulness of the antibiotic agent. This has been demonstrated experimentally by Verwey and Miller[12] wherein they determined the dosage of penicillin which, administered intramuscularly every six hours, would protect

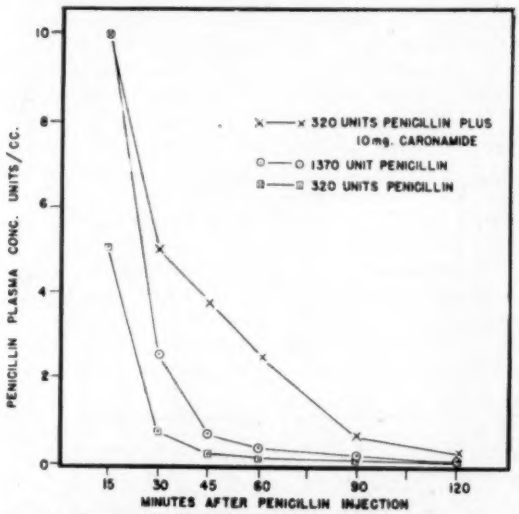


Fig. 8—The penicillin plasma concentration-time curves when penicillin was administered intramuscularly to 20-Gm. mice, alone and with the simultaneous oral administration of caronamide.

50 per cent of their mice infected intraperitoneally with either type 1 pneumococci or *Eberthella typhosa*. Their data on the survival percentages of mice infected with type 1 pneumococci and receiving penicillin with caronamide or penicillin alone are reproduced in table 1. The penicillin was administered intramuscularly every six hours for nine doses, and the caronamide was given in a total dosage of 10 mg. orally at the time of the penicillin injection. Whereas the protective dose 50 for penicillin alone was 55 units administered every six hours, the protective dose of penicillin administered with caronamide was 5 units every six hours. This amounted to an elevenfold advantage for the coadministration of penicillin with caronamide.

It is well known that penicillin is less effective in the treatment of typhoid infections than in the treatment of pneumococcic infections. Table 2, which is taken from their data, illustrates survival percentages of mice infected with *E. typhosa* and receiving penicillin with caronamide or penicillin alone. The modes of administration and the dosage schedule were the same as in the previous illustration. The protective dose 50 for penicillin alone was 1,900 units. When penicillin and caronamide were administered at the same time, the dosage of penicillin necessary to protect 50 per cent of the animals was 515 units. This amounts to 3.7-fold advantage to the coadministration of penicillin with caronamide.

The advantage which accrues to the use of penicillin with caronamide is not due to a synergistic effect of the two agents but is attributable to the elevation and prolongation of the penicillin plasma concentration in the presence of caronamide. This has been demonstrated graphically by Verwey and Miller[12] in figure 8. In the lowest of the three curves in this figure, is recorded the falling plasma concentration in response to the intramuscular administration of 320 units of penicillin to mice. The intermediate curve represents the dosage response following the intramuscular injection of 1,370 units of penicillin. The uppermost curve represents the blood level response to 320 units of penicillin administered intramuscularly, plus 10 mg. of caronamide administered orally. It is evident that the maximal plasma concentration obtainable by the coadministration of 320 units of pencillin with caronamide was equivalent to that obtained by the administration of 1,370 units of penicillin alone. Moreover, when penicillin was administered with caronamide, the antibiotic dosage response curve remained elevated for a much longer period of time, so that the area under the curve was considerably greater than when the larger unitage of penicillin was administered. This may be considered an additional therapeutic advantage of the use of penicillin with the adjuvant over that obtained when penicillin is administered alone in a dosage sufficient to equal the maximal plasma concentration attained with the aid of caronamide.

SUMMARY

From the data that have been presented, it is apparent that the clearance of penicillin approximates the renal plasma flow. Caronamide administered orally or intravenously to dog and mouse is capable of inhibiting the excretion of penicillin by the renal tubules. This inhibitory effect results in an increased plasma concentration of penicillin. Thus, the dosage of penicillin necessary to cure experimental infection in the dog and mouse was 3.7 to 11 times as great when penicillin was injected alone as when it was administered with an oral dose of caronamide.

References

[1]Beyer, K. H., Peters, L., Woodward, R., and Verwey, W. F.: Enhancement of Physiological Econ-

omy of Penicillin in Dogs by Simultaneous Administration of Para-Aminohippuric Acid. J. Pharmacol. and Exptl. Therap., *82*, (1944): 310-323.

[2]Rantz, L. A., Kirby, W. M. M., and Randall, E.: Absorption and Excretion of Penicillin Following Continuous Intravenous and Subcutaneous Administration. J. Clin. Investigations, *23*, (1944): 789-794.

[3]Beyer, K. H., Woodward, R., Peters, L., Verwey, W. F., and Mattis, P. A.: Prolongation of Penicillin Retention in Body by Means of Para-Aminohippuric Acid. Science, *100*, (1944): 107-108.

[4]Mokotoff, R., Brams, W., Katz, L. N., and Howell, K. M.: Treatment of Bacterial Endocarditis with Penicillin: Results of 17 Consecutive Unselected Cases. Am. J. Med. Sci., *211*, (1946): 395-416.

[5]Avery, N. L., Jr., Mayer, O. B., and Nelson, R. C.: Massive Doses of Penicillin in Treatment of Subacute Bacterial Endocarditis. Ann. Int. Med., *24*, (1946): 900-904.

[6]Loewe, L., Rosenblatt, P., and Alture-Werber, E.: Refractory Case of Subacute Bacterial Endocarditis Due to Veillonella Gazogenes Clinically Arrested by Combination of Penicillin, Sodium Para-Aminohippurate, and Heparin. Am. Heart J., *32*, (1946): 327-338.

[7]Sprague, J. M., Ziegler, C. Miller, C., and Cragoe, E. J.: (to be published).

[8]Richardson, A. P., Miller, I., Schumacher, C., Jambor, W., Pansy, F., and Lapedes, D.: Physiological Disposition of Penicillin G and K in Dogs. Proc. Soc. Exptl. Biol. and Med., *63*, (1946): 514.

[9]Beyer, K. H., Miller, A. K., Russo, H. F., Patch, E. A., and Verwey, W. F.: The Inhibitory Effect of Caronamide on the Renal Elimination of Penicillin. Am. J. Physiol., *149*, (1947): 355.

[10]Beyer, K. H., McKinney, S. E., Tillson, E. K., and Green, C. W.: J. Pharmacol. and Exptl. Therap., *91*, (1947): 263.

[11]Beyer, K. H., Russo, H. F., Patch, E. A., and Tillson, E. K.: J. Pharmacol. and Exptl. Therap., *91*, (1947): 272.

[12]Verwey, W. F., Miller, A. K.: Effect of Caronamide upon Penicillin Therapy of Experimental Pneumococcus and Typhoid Infections in Mice. Proc. Soc. Exptl. Biol. and Med., *65*, (1947): 222.

DISCUSSION

DR. ISAAC E. HAYES (Waterloo, Iowa).—Were any tests run on the urine from these animals for glucuronic acid?

DR. BEYER.—Yes, some dogs showed glucuronic acid excretion.

DR. HAYES.—Do you know whether that is the nonfermental substance that you were referring to?

DR. BEYER.—I have tried to be very cautious about that because I don't know whether there are any other such substances.

DR. HAYES.—The penicillin sometimes stimulates it. The second question: Has any work been done with the blood concentration of any of the amino acids, alanine particularly, which increase the capillary bed of the kidney and decrease the filtration pressure of the kidney with regard to maintaining high levels in the blood stream?

DR. BEYER.—No. We have done some alanine clearances, but never in this regard.

DR. CANDLIN (Minn.).—Has any work been done to further prolong the therapeutic effect? Would that be possible?

DR. BEYER.—Clinical work along those lines has been done, yes. There are two different types of inhibitory effects, one with the Romansky formula, penicillin, beeswax, and oil, and one inhibiting the diffusion of penicillin from the site of injection. So from the given unitage of penicillin, the plasma concentration does not go up as high as if penicillin were administered alone, but the distribution is prolonged because it is being allowed to diffuse from the site of injection over a period of time. There is not a very high plasma concentration, but there is a much longer one. The beeswax and oil does not inhibit the tubular excretion and has no effect on the excretion of penicillin. When administered in oil, penicillin is excreted just as fast as given. With the caronamide, the effect is the opposite. There is no interference with the absorption of the penicillin from the site of injection, so tremendous increases in the plasma concentration of penicillin are obtained as well as a prolongation of the blood level.

DR. CANDLIN.—How expensive is that?

DR. BEYER.—I don't know. It isn't on the market.

DR. JAMES V. MCCAHON (Downington, Pa.).—When penicillin is injected into the udder, is it eliminated into the kidneys?

DR. BEYER.—That which is absorbed, yes.

DR. MCCAHON.—What percentage would be absorbed?

DR. BEYER.—I am afraid I can't answer that. Maybe someone here can.

CHAIRMAN GWATKIN.—Any further questions?

DR. MCCAHON.—Is that insoluble? Is it insoluble in oil and therefore nonabsorbable when injected locally into the tissue. Would it remain there or would it be absorbed?

DR. BEYER.—You are talking about caronamide?

DR. MCCAHON.—Caronamide.

DR. BEYER.—We have not administered caronamide by that route. As the acid, it is not very soluble. Actually, when caronamide is administered orally, it is given as the acid, and the body takes it up like that, but if it is dropped in a test tube of water, the acid, *per se*, doesn't go into solution unless it is made slightly basic.

The way we usually prepare the solution is to put it in solution and add sodium bicarbonate and then bring the pH down to whatever we want. It won't come back out of solution until it is down below pH 6. Both the acid and the sodium salt are absorbed when administered orally.

DR. MCCAHON.—Would that decrease the number of times you would have to administer penicillin? It simply economizes on the amount of penicillin necessary, and what we are interested in particularly would be to decrease the interval at which we would have to employ penicillin therapy. If one could administer penicillin, oil, and wax every 24 hours, one would get a full absorption. Then that would really be what we are after.

DR. BEYER.—Caronamide does decrease the rate of excretion of penicillin, that is, over penicillin itself. You get the tremendous plasma concentration over a period of time. You wouldn't want to administer the material intramuscularly together with oil and the like because of the masses of material that would be injected. We do it occasionally, but we don't make a practice of it. In other words, it can be given orally, so we don't bother about putting a large amount of it in subcutaneously. It is very well absorbed.

Brucella Chemotherapy
I. Studies of the Effect of Para-Aminobenzoic Acid on Brucella in Vitro

CORNELIA M. COTTON, Ph.D., and ROBERT E. SWOPE, V.M.D.
College Park, Maryland

PARA-AMINOBENZOIC acid, commonly referred to as PABA, has given evidence during the last few years of a wide range of activity. Since the identification of PABA as a growth factor for many bacteria and a vitamin for plants and animals, as well as the discovery by Woods[1] in 1940 of the antagonistic action between PABA and sulfanilamide, a great deal of investigation has been conducted on the various uses for this substance.

As early as 1940, several workers,[2,3,4] in studying PABA as a growth factor for bacteria, suggested that large quantities of the acid inhibited the growth of a wide variety of organisms and noted that its effectiveness as a growth factor ceased when in concentrations of over 2 mg./100 cc.

In 1942, Snyder, Maier, and Anderson[5] used PABA effectively in treating murine typhus in white mice, and, subsequently, a number of rickettsial diseases have been shown to respond to the same type of treatment to a greater or lesser degree. German workers[6] report no inhibition of the growth of viruses, trypanosomes, spirochetes, malarial parasites, or *Entomoeba histolytica* when treated with PABA.

Whitney and Anigstein[7, 8] report a marked decrease in the bacteria of the guinea pig intestine after treatment with PABA by mouth for eighteen hours. They found, however, that the flora returned to normal after prolonged treatment with the drug.

Rosenblum and Fraser[9] appeared to have had considerable success in treating 9 human cases of acute rheumatic fever with the drug, although previously Dry, Butt, and Scheifley[10] reported that, when used alone, it apparently exerted no effect for the time it was administered.

In view of the generally promising results of some of these studies in the use of PABA, a series of experiments was inaugurated to seek to determine the *in vitro* effects of the chemical on Brucella.

METHODS

Observations of the effects of both PABA and its sodium salt (NaPAB) on the three species of Brucella were made in these studies on both solid and liquid mediums. Two strains of each of the three types of Brucella were used for every *in vitro* study, and each was tested on tryptose agar, with tryptose broth, distilled water, and the blood of horse, cow, sheep, hog, and man. The organisms included:*

Brucella abortus, strains 2308 and 2016.
Brucella suis, strains 2872 and 1942 (2).
Brucella melitensis, strains G 2518 and Henry.

The numbers of organisms used per tube varied from 125 million to 900 million. The results herein given are on 300 million organisms per tube, or approximately 46 million organisms/cc., as measured with the Coleman spectrophotometer unless otherwise stated.

Nine concentrations of PABA or NaPAB, ranging from 3 to 20 mg./cc., were used in each experiment. The amounts were weighed and washed into each culture tube with 1 cc. of distilled water.

Because PABA is not readily soluble in water, a number of methods were tried to accurately determine the smaller amounts used, such as dissolving in acetone, ether, chloroform, measuring and evaporating to dryness, and redissolving in the experimental liquids. None of these techniques proved entirely successful, and weighing was finally used. This undoubtedly caused a great number of inaccuracies, especially in the lower concentrations. The sodium salt of PABA, being quite soluble in water, was placed in the culture tubes in solution, and enough distilled water was added to make a total volume of 1 cc. in each tube.

The tubes containing the PABA or its

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Brucellosis Research, Live Stock Sanitary Service, Maryland State Board of Agriculture, University of Maryland, College Park.

*Cultures were supplied by the Bureau of Animal Industry, Beltsville, Md.

sodium salt were autoclaved, and the liquid medium to be used was sterilized separately and added to each tube in 5-cc. amounts. Organisms were added in distilled water suspension of 0.5 cc. just before incubation was begun. Every experimental tube contained 6.5 cc. Transfers from all tubes were made daily to tryptose agar. When no growth occurred on the agar, the plates were incubated ten days before they were finally discarded, to allow for any bacteriostatic effect which appeared to be present to a marked degree.

Results

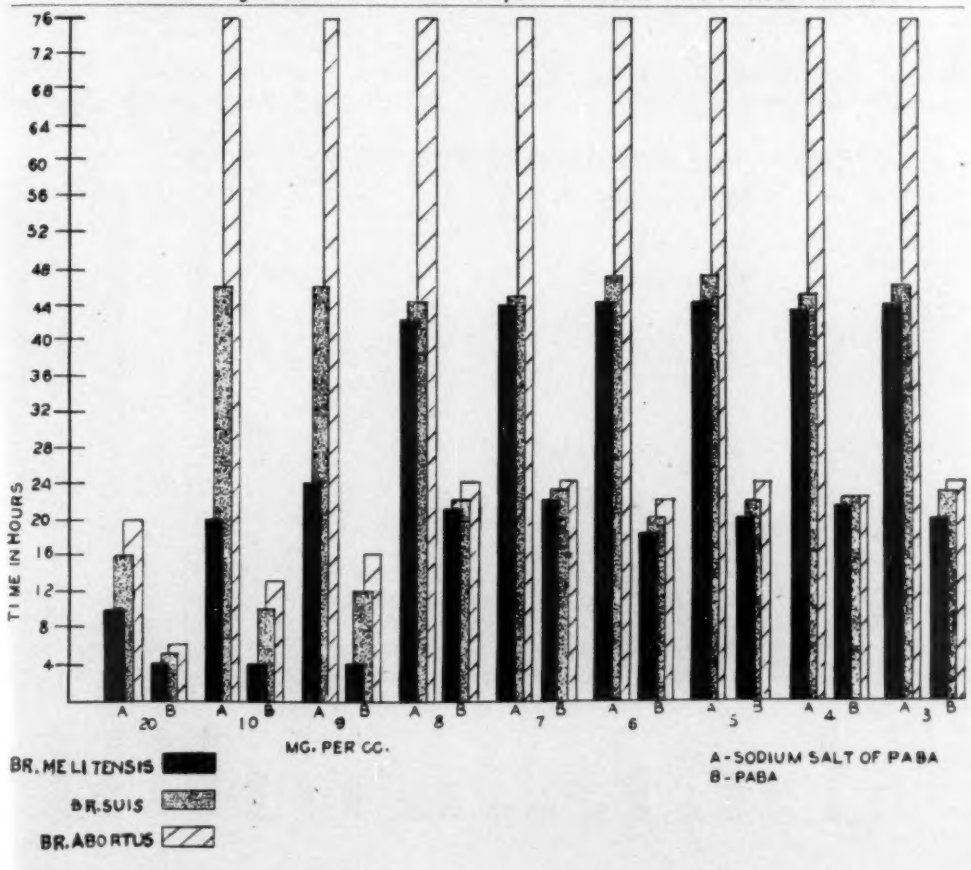
Two mg./cc. of PABA completely inhibited the growth of *Br. abortus*, *Br. suis*, and *Br. melitensis* on tryptose agar, while 5 mg./cc. of the sodium salt was necessary to prevent growth on the same medium. Lower concentrations of the drug did not produce complete inhibition, although the growth of the organisms was slowed by 1-mg. concentrations of it. The pH was adjusted to 6.8 after the addition of the PABA or its sodium salt.

Growth Inhibition of Organisms when Suspended in Distilled Water—Table 1 shows in detail the average death times when the organisms were suspended in distilled water and treated with PABA and NaPAB. One mg./cc. of PABA killed 1/100 as many *Br. abortus* in seventy-two hours as did the higher concentrations but was ineffective on larger numbers of organisms.

When the number of organisms per tube was trebled, the killing time was raised by six hours, while halving the number of organisms per tube only slightly reduced the speed of death in all concentrations of PABA and NaPAB. Variations in temperature changed the killing rate, 37 C. being much more effective than room or refrigerator temperatures.

Growth Inhibition of Organisms in Tryptose Broth.—High concentrations of PABA

TABLE I—Average Death Rate of Water-Suspended Brucella when Treated with PABA



in tryptose broth produced death of all Brucella relatively rapidly, but in lower concentrations the sodium salt was more effective. It must be remembered that the growth of the organisms is stimulated in broth and, consequently, the concentration of organisms varies with the amount of growth which takes place before the action of the PABA or its sodium salt takes effect. It is interesting to note that here, as well as in distilled water, *Br. melitensis* was affected by PABA or NaPAB more rapidly than either *Br. abortus* or *Br. suis*.

There was little difference in the killing time of *Br. melitensis* and *Br. suis* when treble the number of organisms was used, but this increase in numbers slowed the death time materially for *Br. abortus*.

After eight days, 3 and 4 mg./cc. of PABA noticeably inhibited growth of *Br. melitensis*, but through thirty-five days of the experiment some growth was perceptible.

Concentrations of 3, 4, and 5 mg. did not produce sterility in *Br. suis* but inhibited, so that only slight growth on the transfers was noticeable.

Table 2 shows the death times in days of the various organisms in broth.

Growth Inhibition of Organisms in Animal Blood.—When animal blood was used as a medium in testing the effects of PABA on Brucella, a large percentage of the cultures treated with PABA or NaPAB were negative in six to thirty-five days.

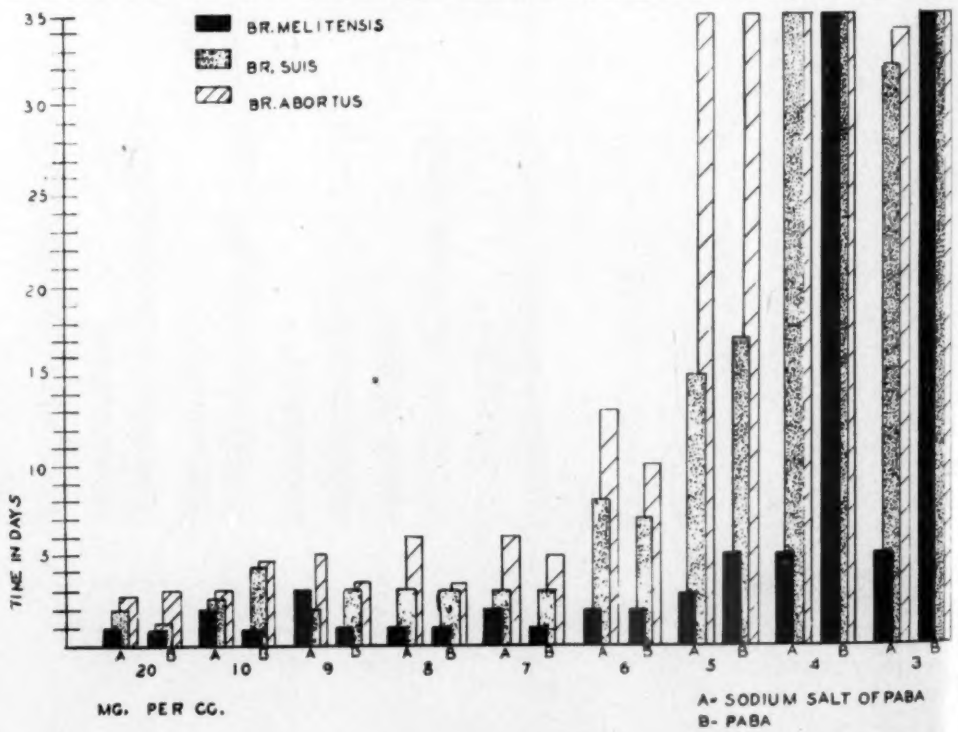
Table 3 indicates the results obtained when using the blood of cattle as a carrying medium with several concentrations of *Br. abortus* organisms. Four and 5 mg./cc. did not kill all the organisms, although there appeared to be considerable evidence that most of the bacteria were dead. Three and 4 mg./cc. of the NaPAB were not bactericidal at forty-one days.

When using the blood of horses as a medium with *Br. abortus* organisms, the results shown in table 4 were seen. The acid PABA did not entirely kill organisms in any concentration during the experiment, although there appeared to be considerable inhibition of growth.

Various concentrations of PABA and NaPAB were used in experiments on *Br. suis* suspended in hog blood. The results are presented in table 5.

Lower concentrations of both PABA and NaPAB had little apparent effect on the or-

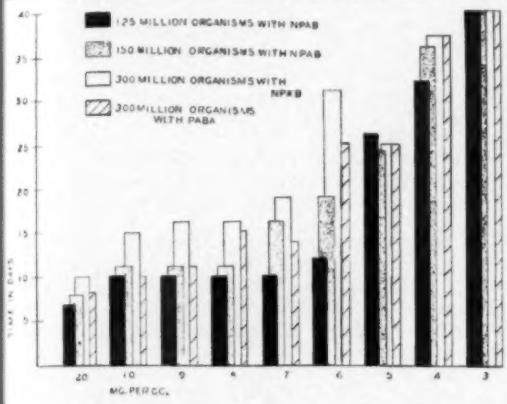
TABLE 2—Average Death Rate of Broth-Suspended Brucella Treated with PABA



ganisms for the duration of the experiment.

The results of the use of PABA and NaPAB on *Br. melitensis* in sheep blood are shown in table 6.

TABLE 3—Comparative Death Rates of Three Concentrations of Brucella Abortus Suspended in Bovine Blood


125 MILLION ORGANISMS WITH NPAB
150 MILLION ORGANISMS WITH NPAB
200 MILLION ORGANISMS WITH NPAB
200 MILLION ORGANISMS WITH PABA
MG. PER CC.

All human blood used was citrated; and it is highly probable that this may have had considerable effect on the killing times, since these periods were much longer than when other animal blood was used. Unneutralized

TABLE 4—Effect of Neutralized PABA on Brucella Abortus Organisms Used with Horse Blood

Concentration (mg.)	Sterile culture (days)	Bacteriostatic effect (days)
20	8	
7	31	
4		42
3		42

PABA had no bactericidal effect on any of the 3 types of Brucella, even after forty-two days, although there was some inhibition of growth. Table 7 shows the average death rates of the 3 types of organisms in citrated human blood.

The control tubes of all experiments showed good growth on transfer for as long as the experiment continued.

It is interesting to note that the colonies of Brucella produced after the prolonged action of PABA or its sodium salt on Brucella differed markedly from the normal in appearance, and these changes seemed to be consistent in all of the work. Their pathogenicity and resistance to PABA have not yet been determined.

SUMMARY

1) Two mg./cc. of para-aminobenzoic acid completely inhibited the growth of *Brucella melitensis*, *Brucella suis*, and *Brucella abortus* on tryptose agar, while 5 mg./cc. was necessary to produce complete inhibition with the sodium salt of PABA.

TABLE 5—Effect of PABA and NaPAB on Brucella Suis Suspended in Hog Blood

	Concentration (mg.)	Sterility reached (days)
PABA	20	22
	9	30
	8	35
NaPAB	20	21
	10	25
	9	29
	8	35

2) *Brucella melitensis*, *Br. suis*, and *Br. abortus* suspended in distilled water were killed in all concentrations of PABA from 3 to 20 mg./cc. within eighteen hours, when there were 46 million organisms/cc. in suspension. The higher concentrations produced sterility in less than four hours.

TABLE 6—Effect of PABA and NaPAB on Brucella Melitensis in Sheep Blood

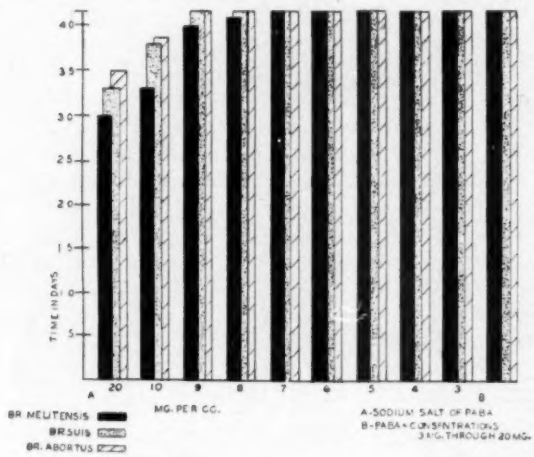
	Concentration (mg.)	Sterility reached (days)	Little apparent effect (days)
PABA	20	20	
	8	31	
	6	35	
NaPAB	20	8	
	7	18	
	6	25	
	4		42
	5		42

The sodium salt of para-aminobenzoic acid (NaPAB) affected the organisms more slowly. Under the same conditions *Br. melitensis* and *Br. suis* were killed in all concentrations within forty-eight hours and *Br. abortus*, within seventy-two hours.

3) In tryptose broth, higher concentrations of PABA produced sterility of the cultures rapidly, but in lower concentrations NaPAB was more effective. *Brucella melitensis* was killed more rapidly than *Br. suis*

or *abortus*. The death times were lengthened from a few hours to two or three days over those in distilled water.

TABLE 7—Average Death Rate of Brucella Suspended in Citrated Human Blood and Treated with PABA



4) In all blood, the sodium salt of PABA was much more effective than the acid. The death times were much lengthened over these found in distilled water and broth. Variations of from eight to thirty-five days were noted and in some bloods, the lower concentrations of 3, 4, and 5 mg./cc. were not effective killing agents.

References

[1]Woods, D. D.: The Relation of Para-Aminobenzoic Acid to the Mechanism of Action of Sulfanilamide. British J. Exptl. Path., *21*, (1940): 74-90.

[2]Rubbo, S. D., and Gillespie, J. M.: Para-Aminobenzoic Acid as a Growth Factor. Nature, *146*, (1940): 838-39.

[3]Selvag, M. G., and Shelbourne, M.: The Respiration of Streptococcus Pyogenes and Pneumococcus Type 1. J. Bact., *43*, (1942): 447-462.

[4]Rubbo, S. D., Maxwell, M., Fairbridge, R. A., and Gillespie, J. M.: The Bacteriology, Growth Factor Requirements and Fermentation Reactions of *Cl. Acetobutylicum*. Austral. J. Exptl. Biol. and Med. Sci., *19*, (1941): 195-198.

[5]Snyder, J. C., Maier, J., and Anderson, C. R.: Report to the Division of Medical Sciences, National Research Council, Dec. 26, 1942.

[6]Mudrow, Lilly, and Bach, Marianne: The Antagonism of the Sulfonamides and Para-Aminobenzoic Acid in Experimental Infections with Spirochaetes, Spirillias and Viruses. Z. Immun., *104*, (1943): 463-173. Chem. Zentralbl., 2, (1944: 1936).

[7]Whitney, D. M., and Anigstein, L.: The Effect of Para-Aminobenzoic Acid on the Intestinal Flora of Guinea Pigs. J. Bact., *52*, (1946): 400.

[8]Whitney, D. M.: Further Observations on the Intestinal Bacteria of Guinea Pigs Fed with Para-Aminobenzoic Acid. Texas Rept. Biol. and Med., *5*, (1947): 63-73.

[9]Rosenblum, H., and Fraser, L. E.: Effect of Para-Aminobenzoic Acid on Fever and Joint Pains of Acute Rheumatic Fever. Proc. Soc. Exptl. Biol. and Med., *65*, (1947): 178-180.

[10]Dry, T. J., Butt, H. R., and Scheifley, C. H.: The Effect of Oral Administration of Para-Aminobenzoic Acid on the Concentration of Salicylates in the Blood: Preliminary Report. Proc. Staff Meet. Mayo Clinic, *21*, (1946): 497-504.

Brucella Chemotherapy. II. Para-Aminobenzoic Acid in the Treatment of Experimental Brucellosis in Guinea Pigs

CORNELIA M. COTTON, Ph.D., and ROBERT E. SWOPE, V.M.D.

College Park, Maryland

PARA-AMINOBENZOIC acid (PABA) has been studied extensively as an antagonistic agent in the treatment of various diseases with the sulfa drugs and has received some attention as a vitamin and hormone. It was not until 1942 that Snyder, Maier, and Anderson[1] discovered the beneficial effect of PABA in the treatment of murine typhus in white mice. Their work was not published at the time because of its importance in the war effort. Meanwhile, Grief, Pinkerton, and Moragues,[2] quite independently, made similar observations on murine typhus, using the yolk sacs of eggs for study. In 1945, they published similar results on epidemic typhus.[3] The use of PABA in the treatment of Rocky Mountain spotted fever was suggested by Hamilton,[4] who reported that it was more effective in this disease than in typhus. Anigstein and Bader[5] used the drug in experimental spotted fever of guinea pigs. The Rickettsia of scrub typhus has also been shown to be affected to some extent by PABA, as reported in three papers by Snyder, Murray, and Zarefonetes.[6,7,8] Case reports of the several types of rickettsial diseases in human beings are appearing in the literature, showing a general measure of success, especially in Rocky Mountain spotted fever.

Because of the bactericidal effect of PABA on Brucella *in vitro* (as shown in the first paper of this group[11]), and making use of the techniques already worked out by the investigators on rickettsial diseases, a series of experiments was inaugurated to determine the effects of the chemical on brucellosis in guinea pigs.

METHODS

Four groups of experimental guinea pigs were used in the tests. These included animals treated with the drug for seventy-two hours before the infective dose was administered; guinea pigs in which the treatment was begun at the same time the infective inoculum was injected; animals which were infected with Brucella seventy-two hours before treatment was begun; and guinea pigs in which the infective dose was administered two weeks before treatment was started.

The guinea pigs approximated 500 Gm. in weight when infected and were weighed at weekly intervals throughout the experiment. It was obviously important that there should be no question but that the guinea pigs were actually infected with brucellosis, therefore the infective dose was 1½ billion virulent bovine Brucella (strain 2308) injected intraperitoneally into each guinea pig. Forty-eight-hour cultures of the organism, suspended in distilled water, were used, and all measurements of organisms were checked with a Coleman spectrophotometer. The drug was injected subcutaneously, even though investigators of rickettsial diseases[10,11] had found it effective when given by mouth. However, it seemed that the amount of drug administered could be more easily regulated in guinea pigs when given subcutaneously. Preliminary experiments indicated that PABA was not well tolerated by the animals when given in large quantities, and these findings are confirmed in the literature. Therefore, the drug was administered in a solution of sodium bicarbonate, which was added in sufficient quantities so that the resulting solution was neutral to litmus paper. The chemical reaction produced the sodium salt of PABA (NaPAB). In order that there should be no deterioration on standing, sufficient salt was prepared for only one series of injections at a time.

Blood was collected before, during, and after treatment for opsonocytophagic and agglutination tests, as well as for the determination of blood levels. The guinea pigs were all killed thirty-eight days after the injection of Brucella and were observed for gross lesions. The spleen, liver, kidneys, heart, lungs, and reproductive organs of each animal were cultured on tryptose agar and broth.

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Brucellosis Research, Live Stock Sanitary Service, Maryland State Board of Agriculture, University of Maryland, College Park.

These cultures were incubated two weeks before being discarded as negative.

EXPERIMENTAL

Experiment 1.—The 25 guinea pigs were divided into five equal groups. Five animals were placed in a control group; and the other 20 were divided into four experimental groups, which were treated every four hours with 5 cc. of 1 per cent NaPAB. Group 1, the controls, showed enlarged nodular spleens at autopsy, and Brucella were isolated from the liver and spleen of each of the animals. Group 2 was treated for three days before receiving the infective dose and for one day following it. Three of the 5 guinea pigs survived the experiment, and none of these showed typical lesions of brucellosis. One of them did give a positive spleen culture. Group 3 received the infective dose and the first treatment simultaneously. Three of the 5 showed no lesions and gave no positive cultures. Two animals died before the experiment was completed, but no Brucella were isolated from them. Pigs of group 4 were infected three days before treatment was begun. All showed spleen enlargement with small lesions, and Brucella were isolated from 3 of the 5. The guinea pigs of group 5 received the infective dose fourteen days before treatment was begun. One animal died during the first treatment, and Brucella were isolated from every organ. On autopsy, thirty-eight days after infection, the 4 remaining guinea pigs all showed enlarged and nodular spleens, and from 3 of the spleens Brucella were isolated.

The average titer of all guinea pigs in experiment 1 at two weeks was 1:200; at four weeks, 1:500; and at five weeks, 1:200. There was no appreciable difference in the titers of the groups. The blood level of PABA ranged from 7 to 14 mg./100 cc. in this experiment, depending on the time after treatment that the blood samples were taken.

While the results in this experiment were inconclusive, there did seem to be enough evidence of bacteriostatic effect to warrant further work along the same lines.

Experiment 2. — Since the titers of the guinea pigs in experiment 1 did not indicate a severe form of the disease, the *Brucella abortus* culture used in this experiment, though of the same strain, was one that had more recently been animal-passed and appeared to be more virulent. The NaPAB was given subcutaneously every four hours in doses of 5 cc. of a 5 per cent solution, and the length of the treatment was materially increased. Other conditions were the same as those in the first experiment. Group 1 controls developed titers of 1:1,000 and 1:2,000 within the three weeks, and these remained until they were killed in thirty-eight days. Abscessed lesions were present in the spleen, liver, and kidneys of every animal in the control group and in the heart, lungs, or reproductive organs of some of them.

The pretreated guinea pigs of group 2 received NaPAB for three days before the infective organisms were administered and for seven days after. Two of the 5 animals showed slight enlargement of the spleen at autopsy, and from 4 of the 5, Brucella were obtained from the spleen but from no other organ. One guinea pig was negative for Brucella on culture.

Animals of group 3, which received the first treatment at the same time the infective dose was given, did not survive the treatments well, and, hence, they were discontinued on the seventh day. Two of the 5 animals in this group died during the treatment, and 2 of the remaining were negative and 1 positive on culture.

The animals in group 4 (infected three days before administration of the drug) were treated for fourteen days. There were no visible lesions on autopsy, but there was slight spleen enlargement in all animals with some hemorrhagic appearance. Two animals were negative, and 3 had positive spleens on culture.

Guinea pigs of group 5 (infected fourteen days prior to treatment) were treated sixteen days. Spleens were all normal in gross appearance, but Brucella were isolated from 3 of the animals—from the kidney of 1 and the spleen of the other 2. Two animals were entirely negative.

The titers produced in experiment 2 were much higher than those in the previous experiment. They averaged 1:500 in all the groups two weeks after infection, and 1:1,000 or greater at the time the animals were sacrificed. The blood levels ranged from 18 to 42 mg./100 cc. during the treatments.

PABA did appear to overcome the infection in some of the treated animals; and there was considerable evidence to show that even where Brucella were isolated, the colonies were few and scattered, in contrast to the heavy growth on the control plates.

The results of the previous experiment seemed to be suggestive enough to warrant larger doses of the drug, so it was decided to administer PABA over a longer period of time in the third experiment.

Experiment 3. — Some changes were made in the number of animals used in the groups of the third experiment. Group 2 was eliminated from the study, and 12 guinea pigs were

used in each of groups 3, 4, and 5, with 18 animals in the control group. Considerable difficulty was encountered in this experiment, since many of the guinea pigs were in poor physical condition when they arrived and died during the experiment. The same treatment was used as in experiment 2, 5 cc. of a 5 per cent solution of NaPAB every four hours; but in this experiment it was continued for twenty-one days. The control group at autopsy was heavily infected, and Brucella were obtained from every organ cultured in 8 pigs, and from at least 3 organs in each of the remaining animals. Such a large number of guinea pigs of group III (where infection and treatment took place simultaneously) died early in the experiment that the results were inconclusive. One guinea pig, however, was interesting. It carried a litter of 3 young to term through twenty-one days of treatment and for thirteen days past the treatment period. Although the spleen of this animal was enlarged and roughened, the uterus was the only organ from which Brucella were isolated; and the organisms obtained were atypical.

Guinea pigs of group 4, infected three days before treatment was begun, responded to twenty-one days' treatment in a very satisfactory manner. Five of the 12 pigs died early in the treatment from a variety of causes. The remaining 7 were sacrificed thirty-eight days after infection and showed no lesions; no Brucella were obtained from any organ. The spleens of 2 animals were very slightly enlarged and somewhat nodular, but 5 pigs showed smooth and normal appearing spleens.

Those animals infected two weeks before treatment was begun, group 5, showed almost as satisfactory results from twenty-one days' treatment with NaPAB as group 4. Two of those pigs died early in the treatment and gave positive cultures, but 10 were sacrificed at thirty-eight days. Eight of the 10 were negative on culture for brucellosis, and 2 gave a few atypical colonies from the spleens. These organisms were positive to the agglutination test for Brucella. The spleens of the positive guinea pigs were normal in appearance, as were the spleens of 6 other animals. Two pigs, from which no organisms were cultured, showed slightly enlarged and nodular spleens, however.

The titers of these guinea pigs in experiment 3 also were above 1:500 in all groups two weeks after infection, and 1:1,000 or greater at the time they were killed. Blood levels ranged from 15 to 45 mg./100 cc. during the treatments.

Discussion

In these groups of treated animals, there seems to be no doubt as to the bactericidal action of the sodium salt of PABA on the organism. Lesions were entirely absent in the treated guinea pigs; and even when Brucella were isolated, not more than two to six colonies appeared on a plate, in contrast to the heavy growth on the control plates.

All group 1 control guinea pigs lost weight averaging 100 Gm., while the average loss of the experimental pigs was 49 Gm. Weight loss early in the experiment was marked in all groups, but the treated pigs gained rapidly as treatment progressed. The treatment apparently stimulated the appetite to a marked degree, particularly after the animals became accustomed to the frequent handling.

Some few of the deaths among the treated pigs appeared to be due to shock. Such deaths occurred during the first few days of the treatment and followed the injection of the drug within a few minutes. Most of the deaths, however, could be traced to causes other than shock.

The blood levels in guinea pigs do not seem to be very significant, as measured by the Eckert[9] method, as there appeared to be a substance (possibly an organic acid) in normal, untreated guinea pigs, which gave a similar color reaction to PABA. Although the treated guinea pigs showed fairly uniform bloods levels, the untreated animals varied considerably from time to time, often showing very marked color reaction. There would appear, from our results, to be no hindrance in the treated animals, and this may be caused by neutralization of the interfering substance by the sodium salt of PABA. Further work is being done on the causes of this disturbance in the tests in normal guinea pigs. This apparently is the same substance mentioned by Anigstein and Whitney.[10]

Another interesting observation was the change in colonial appearance of the Brucella isolated from the treated animals. The size of the average colony was about ten times larger than the colonies of the strain used for the infective dose. The color and density of the colonies appeared to be similar to those usually noted in Brucella. Such observations were also made on the *in vitro* studies.

SUMMARY

Guinea pigs heavily inoculated with a virulent bovine strain of *Brucella abortus* were treated with the sodium salt of para-aminobenzoic acid. A 5 per cent solution of the sodium salt, administered subcutaneously in 5-cc. amounts every four hours for twenty-one days, appeared to produce sterilization in 100 per cent of the animals when treatment was begun three days after the infective organisms were injected. Where treatment was delayed for two weeks after infection, 80 per cent of the animals were negative to cultural examinations of spleen, liver, kidney, heart, lungs, and reproductive organs.

References

[1]Snyder, J. C., Maier, J., and Anderson, C. R.: Report to the Division of Medical Sciences, National Research Council, Dec. 26, 1912.

[2]Grief, D., Pinkerton, H., and Moragues, V.: Effect of the Enzyme Inhibitors and Activators on the Multiplication of Typhus Rickettsiae. 1. Penicillin, Para-Aminobenzoic Acid, Sodium Fluoride and Vitamins of the B Group. J. Exptl. Med., *89*, (1944): 561-574.

[3]Grief, D., and Pinkerton, H.: Effect of Enzyme Inhibitors and Activators on the Multiplication of Typhus Rickettsiae. 2. Temperature, Potassium Cyanide and Toluidine Blue. J. Exptl. Med., *82*, (1945): 193-206.

[4]Hamilton, R. L.: Effect of Para-Aminobenzoic Acid on the Growth of Rickettsial and Elementary Bodies, with Observations on their Mode of Action. Proc. Soc. Exptl. Biol. and Med., *59*, (1945): 220-226.

[5]Anigstein, L., and Bader, M.: Para-Aminobenzoic Acid, Its Effectiveness in Spotted Fever in Guinea Pigs. Science, *101*, (1945): 591-592.

[6]Snyder, J. C., and Zarefonetes, C. J. D.: Effects of Para-Aminobenzoic Acid in Experimental Tsutsugamushi Disease. Proc. Soc. Exptl. Biol. and Med., *60*, (1945): 115-117.

[7]Murray, E. S., Zarefonetes, C. J. D., and Snyder, J. C.: Further Report on the Effect of Para-Aminobenzoic Acid on Experimental Tsutsugamushi Disease. Proc. Soc. Exptl. Biol. and Med., *60*, (1945): 240-242.

[8]Zarafonetes, C. J. D., and Snyder, J. C.: Immunity Following Para-Aminobenzoic Acid Therapy in Experimental Tsutsugamushi Disease. Proc. Soc. Exptl. Biol. and Med., *61*, (1946): 241-242.

[9]Eckert, H. W.: PABA Determination in Blood and Urine. J. Biol. Chem., *148*, (1943): 197-203.

[10]Anigstein, L., and Whitney, D. M.: Evaluation and Significance of PABA Blood Levels in Guinea Pigs with Reference to Spotted Fever Treatment. Texas Rept. Biol. and Med., *4*, (1946): 338-352.

[11]Cotton, Cornelia M., and Swope, Robert E.: Brucella Chemotherapy. I. Studies of the Effect of Para-Aminobenzoic Acid on Brucella *in Vitro*. Am. J. Vet. Res., *9*, (1948): 164-168.

DISCUSSION

MEMBER.—Did you expose any of these pigs after you treated them and did you find them to be negative with Brucella organism?

DR. COTTON.—No, we didn't because we had to kill them in order to get our results. We were very short of pigs. Had we had more, we could have tried that.

DR. MANTHEI.—I would like to know what effect the drug had on the titers of the animals that had been infected fourteen days. Eight out of 10 were negative; were the titers also negative?

DR. COTTON.—In general, we found there was a slight reduction in the titer, but not enough so that we would really wish to report.

Growth of the Adrenal Cortex in Domesticated Ungulata

HANS ELIAS, Ph.D.
Atlanta, Georgia

SINCE 1902, it has been known, through the investigations by Mulon,[11] that the aging cells of the zona reticularis of the adrenal cortex of the guinea pig are removed through the blood sinusoids which abound in this region. One year later Mulon[12] showed that these disappearing cells are replaced by cells from the zona fasciculata, which in turn are replenished by cells from the zona glomerulosa. Since that time, it has been the general opinion that this cell replacement is accomplished by cell division.

Hoerr[9] determined, again working with guinea pigs, that the site of the mitosis was the outer region of the zona fasciculata, and that occasionally mitoses may occur in the zona glomerulosa. Blumenthal[3] confirmed Hoerr's findings and stated that "the site of mitotic division was confined to an area consisting of the inner portion of the zona glomerulosa and the outer portion of the zona fasciculata."

However, the number of mitoses found by both Hoerr and Blumenthal (1.3 to 4.6 for a longitudinal section of a guinea pig's adrenal gland) could hardly account for the replacement of so many degenerating cells.

Zwemer, Wotton, and Norkus,[15] investigating cat, monkey, man, and unidentified rodents, solved the problem. They concluded "that new glandular cells are derived by morphogenesis from indifferent cells of the capsule."

Bachmann,[1] Gruenwald,[7,16] and Gruenwald and Konikov[8] confirmed this observation for man, rhesus monkey, guinea pig, rabbit, cat, and dog. New evidence for cell replacement from the capsule was added by Elias[5] in a preliminary report describing the growth of the adrenal cortex of the horse, the ox, and the goat.

It was already evident from Gruenwald and Konikov's paper that there is more than one manner in which the undifferentiated cells of the capsule arrange themselves in groups, and that their incorporation into the cortex can occur in more than one way.[8] They classified the different methods of appositional growth into two groups: (1) end to end junction of newly formed cords in the capsule with those of the zona glomerulosa; (2) downward movement of broad cell masses and their final fusion with the cortex. Gruenwald and Konikov,[8] however, say: "The great variability in the structure of the zona glomerulosa in mammals suggests that the number of mechanisms of junction between new and old cortical tissue may not be limited to the two described in the present report. Study of additional material may well reveal new variations of these two types of apposition, or entirely different types."

Indeed, observations[5] on the horse and on the ox showed new types which can hardly be classified in one of Gruenwald and Konikov's types. Also, the manner of apposition described by Bachmann for the cat does not fit either of the two types.[1]

The present observations show several additional modes of cell replacement. However, not only will the various types of apposition be listed, but the structure of the adrenal cortex of the five species of ungulates domesticated in Europe and North America will be described.

It must be noted that former observations, chiefly those by Gruenwald,[16] have shown that embryonic, fetal, and early postnatal development of the adrenal cortex occurs in a way entirely different from the growth of the adrenal in later infancy and adulthood. Only the latter phase of growth, methods of cell replacement in fully developed adrenal glands, is considered in this paper.

Since several methods of appositional growth have been observed in single individuals, and the number of individuals examined is small, it can be assumed that more modes of such growth exist in the species under examination than are listed here.

From the veterinary standpoint, it seems imperative to describe the various species separately. It is also necessary that the structure of the adrenal cortex of each individual species be described, since our past informa-

From the U. S. Public Health Service, Atlanta, Ga.

tion on this subject is scanty and superficial. Thus, the main part of this paper will be divided into five specific chapters, each dealing with one species.

Only the chapter on the horse will be organized in the traditional way. In it, discussions will follow descriptive parts. All the controversial and basic considerations pertaining to the entire study will be dealt with in the chapter on the horse. Since they are applicable to the other species as well, they will not be repeated. For the sake of brevity, observations and their interpretations will be fused during the consideration of the non-equine species.

MATERIALS AND METHODS

The equine and caprine material used was derived from 3 embalmed horses and 2 embalmed goats, *i.e.*, fixation was accomplished by injection, after bleeding, into the carotid artery of a mixture of formalin, glycerin, and carbolic acid, a method which had proved excellent for the instantaneous preservation of finest cellular detail in all parts of the body. The rest of the material was obtained from different abattoirs. Fixation of this material was accomplished in Bouin's solution. Three pigs, 3 calves, and 3 lambs were used. Portions of both adrenals of each animal were cut in serial sections. Serial sections are very essential in this study, for when single sections only were examined, it frequently could not be determined whether certain cell groups were connected with the main body of the gland. Only in the case of the goat, single sections were used.

I) THE HORSE

A) THE STRUCTURE OF THE ZONA ARCUATA

The outer region of the adrenal cortex of the horse has long been termed zona arcuata because of the large, arched figures of which it is composed. Vertical sections through the zona arcuata are shown in figures 1, 2, 3, and 4. Some of the elements are, indeed, arched; others appear in sections as simple bands. Single, vertical sections give little information about the real structure of these elements. In order to arrive at a proper visualization, serial sections in both the vertical and horizontal direction were studied.

Figure 5 is taken from a tangential section through the outer part of the zona arcuata. It shows that many of the units of this zone are closed and rounded. A synthesis of both vertical and tangential sections through this region shows that we deal here with domelike structures. When looking at sections through the deeper layers of the zona arcuata (fig. 6), we see long, curved bands which are not recurrent into themselves. From all these sections, we can conclude that the zona arcuata contains large, folded laminae with the convex surfaces directed toward the capsule, the free borders being in contact with the zona fasciculata. The laminae are of limited extension. Neighboring laminae are not connected with one another. Many of these folded membranes have apical outpouchings in the shape of tall domes. Some of the laminae are pro-

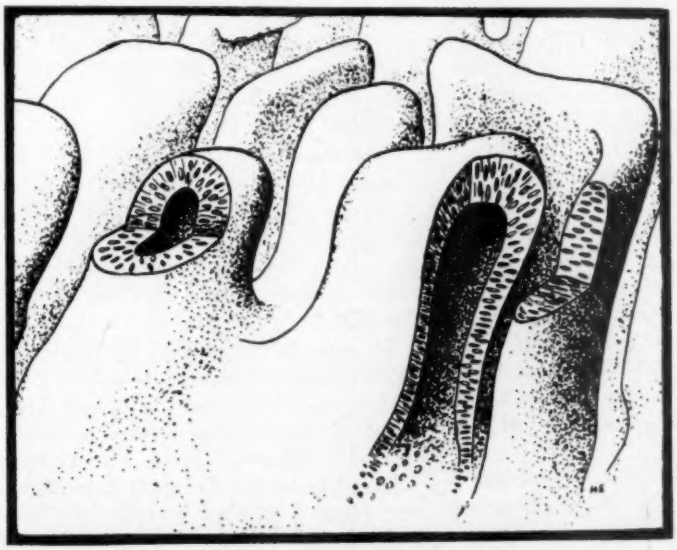

Fig. A—Stereogram of some elements of the zona arcuata of the horse.

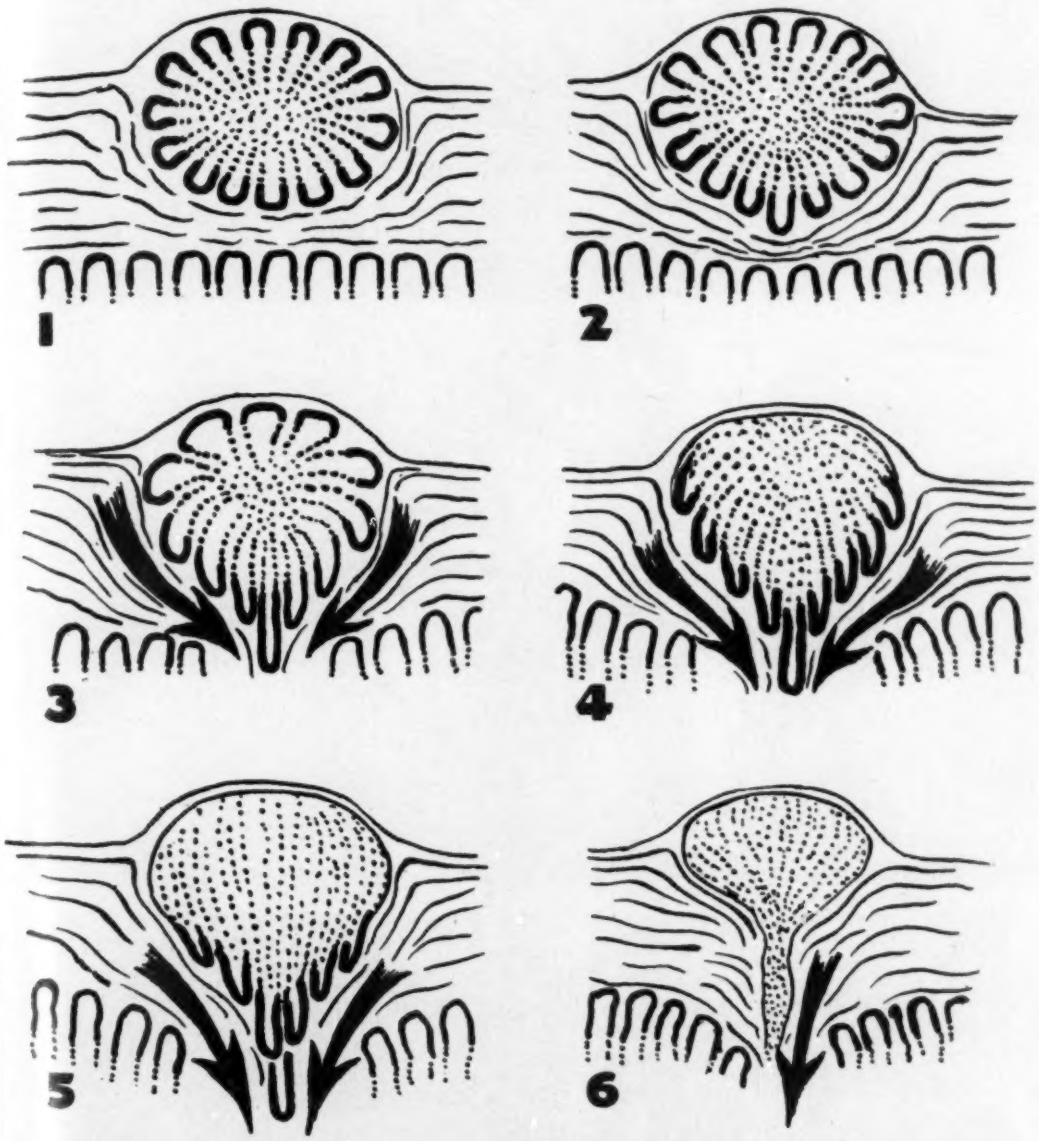

Fig. B—Diagram of the mechanics of incorporation of a nodule into the adrenal cortex of the horse. (Solid black arches, zona arcuata; stippled, zona fasciculata.) (1) Fully developed nodule in capsule. (2) Capsule becomes thinner at point of closest approximation (corresponds to fig. 9). (3) The capsule becomes perforated; the zona arcuata of the nodule begins to be drawn inward. (4) More of the zona arcuata is drawn in. The parietal surface of the nodule is beginning to be denuded of zona arcuata. (5) The greater part of the zona arcuata of the nodule has been drawn in (corresponds to fig. 11). (6) The entire zona arcuata has been incorporated. The zona fasciculata of the nodule is now being drawn in (corresponds to fig. 10).

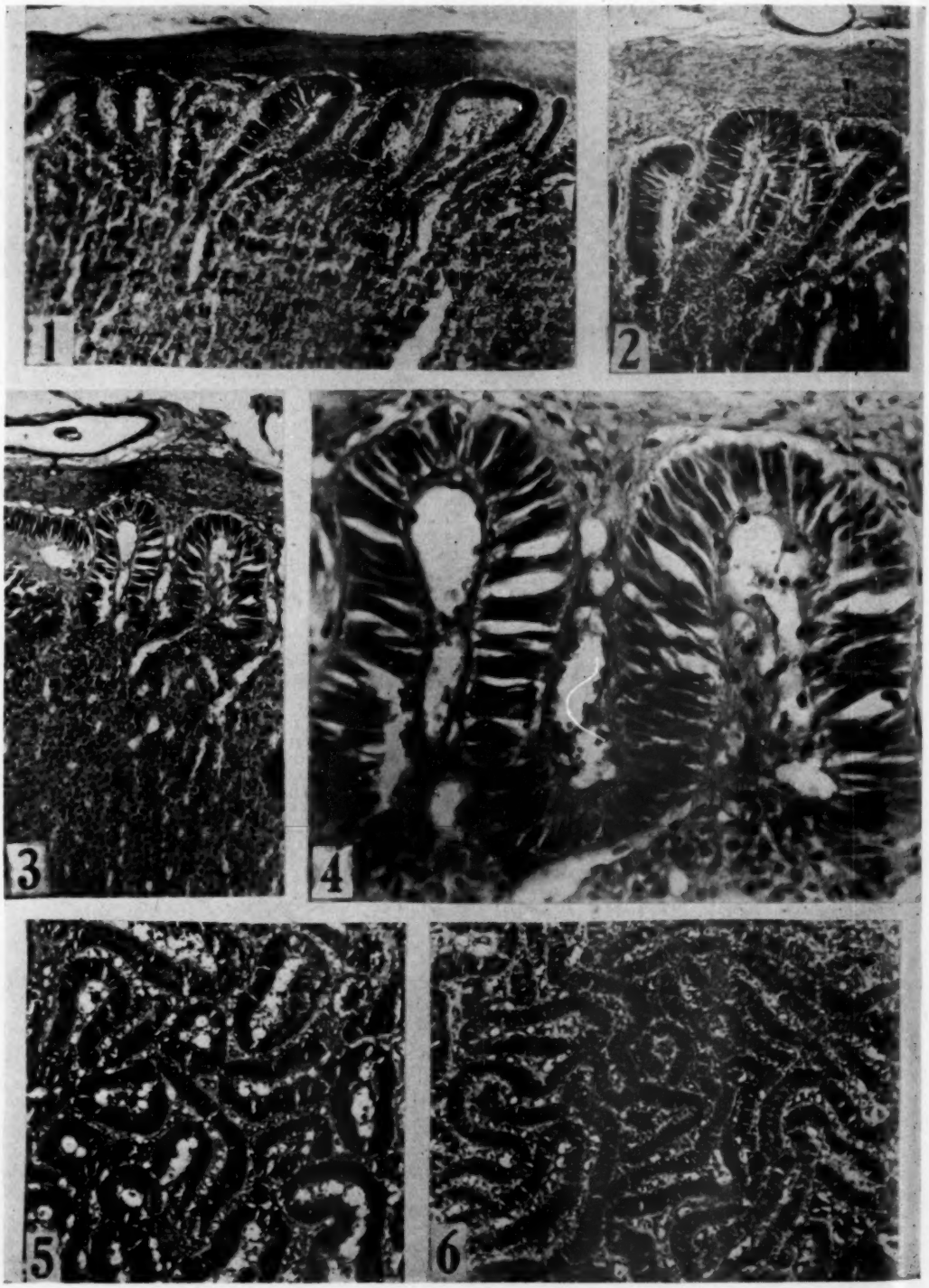

PLATE I

Fig. 1 to 4 are vertical sections through the zona arcuata of the horse, figure 4 being a higher magnified detail from figure 3.

Fig. 5 to 6 are horizontal sections through the zona arcuata of the horse, figure 5 cut at a high level. Figure 6 cut at a low level.

vided with more than one such domelike outpouching (fig. 3 and 4). In figure A, a stereographic reconstruction of some elements of the zona arcuata is given.

Those elements of the zona arcuata which appear as simple bands in vertical sections (fig. 1 and 2) have been interpreted as cords.[8] However, tangential sections of the zona arcuata (fig. 5, just below center) show that they are broad, unfolded sheets, set vertically between the folded lamellae. If cords existed in this region, tangential sections of the cortex would have to show their isodiametrical cross sections, but such sections cannot be found. What we find are long, simple, bandlike sections. Clearly, we can conclude that these are the horizontal sections of vertically standing, simple, nonfolded laminae. Figure A shows, at the right, such a simple, vertical lamina stereographically presented.

In addition to the folded or arched and vertical laminae, flat, horizontal laminae are found (*see* left, fig. 3).

The laminae composing the zona arcuata have the character of a pseudostratified epithelium composed of very tall cells which reach from one surface of the lamina to the other (fig. 4). The laminae are lined on both sides by the endothelium of blood sinusoids or by reticular connective tissue (fig. 4).

B) CORTICAL CELL GROUPS IN THE CAPSULE

1) *Cell Groups of Nodular Character.*—Figure 7 (plate 2) shows groups of cortical cells of varied size and arrangement within the capsule: At 1 we find very small groups of small cells located in rounded tissue spaces. At 2 we see cell groups a little larger and having the shape of lenses or discs. The cells within them are elongated and stand perpendicularly to the plane of the group. They manifest clearly the histologic character of the zona arcuata. At 3, 4, and 5 we see, confined in rounded tissue spaces, progressively larger laminae which are folded. These cell groups constitute cortical nodules consisting wholly of zona arcuata tissue, very similar to some of those described for the cat.[2]

Figure 9 shows a much larger nodule. Its peripheral layer consists of zona arcuata and its core of zona fasciculata. Nodules of this kind have also been described for the cat.[2] As serial sections show, this nodule is entirely separated from the main body of the gland. The section shown in figure 9 contains the point of closest approximation of this nodule to the gland. At one point (lower right) only a very thin, capsular membrane separates it from the cortex. The nodule has a downward bulge, and a lamina of arcuata tissue approaches it from below.

Figure 11 shows a nodule which appears as an inverted sector of the cortex. Its zona arcuata is found in proximity to the main gland. But the cell cords of its zona fasciculata are directed toward the periphery. There exists a narrow strand of zona arcuata tissue connecting the nodule with the zona arcuata of the main gland, through a funnel-like opening, piercing the capsule. Observation of serial sections shows that a cross section through this funnel is circular, the mass within it cylindrical; and the entire nodule is shaped like a mushroom. Bennett,[2] describing similar nodules (with a proximal zona glomerulosa and a distal zona fasciculata) for the cat, did not find their narrow connections with the main cortex, perhaps because he might not have examined serial sections.

Figure 10, finally, shows a mass of zona fasciculata tissue, located at the very periphery of the capsule. This mass is connected with the cortex by means of a strand of zona fasciculata tissue.

DISCUSSION—Bennett[2] assumed that those nodules which he observed in the cat, were small, accessory, cortical glands which remain permanently located in the capsule.

Dagonet[4] had reported the occurrence of such nodules in man. He showed them in connection with the main cortex and others isolated from it. Since he was able to demonstrate many intermediate forms, which fitted into one developmental series, he drew the conclusion that the adrenal cortex gave rise, by budding, to new, independent, accessory adrenal glands. This is an interesting example of how a series of fixed developmental stages can be interpreted in either direction.

The discoveries of Mulon[11, 12] gave evidence that in the adrenal cortex an inward movement of cells takes place. In addition, the observations by Lewis and Pappenheimer[10] and Swinyard[13] have shown that in the human newborn the zona glomerulosa exists, but that the zona fasciculata appears later. This fact shows that the zona glomerulosa (and for that matter, the zona arcuata) cannot arise from the zona fasciculata.

The rôle which the capsule and the outer portion of the glomerulosa play as the matrix for the more centrally located portions of the cortex has also been demonstrated by Williams[14] who transplanted capsule and portions of the adjacent glomerulosa into rats' ears and found that these (autogenous) grafts developed into functional glands containing a zona fasciculata and a zona reticularis. These grafts became established as permanent glands which kept the rats as well as the original cortex alive. Zona reticularis and zona fasciculata grafts, however, had no power of regener-

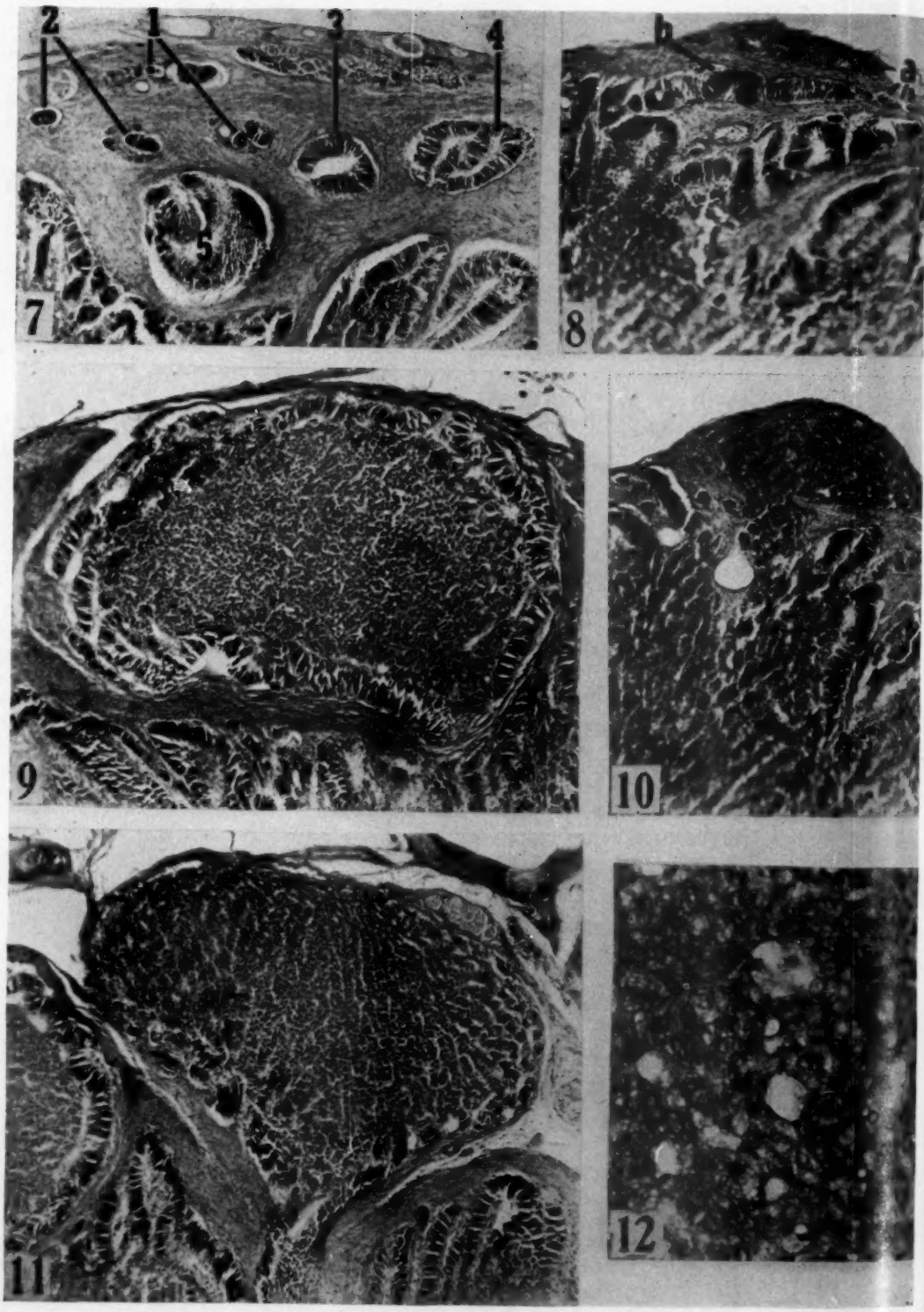

Plate 2

ation. These grafts and their donor-hosts did not survive.

All these new observations indicate that Dagonet was wrong inasmuch as he saw the same series of stages move in the opposite direction. Thus we are ready for a valid interpretation of our observations.

It appears that the cell groups found in figure 7, (plate 2) represent early stages in the development of nodules: Small groups of undifferentiated cells enlarge (1). The cells begin to elongate and arrange themselves perpendicularly to the direction of the long axis of their cell group (2). These cell groups become larger and assume the form of plates. Since these cell plates grow but do not find sufficient space for unhampered extension, they fold themselves (3, 4, 5).

Thus it comes about that nodules of zona arcuata tissue arise within the capsule. These continue to grow, and the zona arcuata tissue of their interior is transformed into secretory tissue having the appearance of the zona fasciculata (fig. 9).

Figure B illustrates, schematically, the subsequent, probable development. Figure B 1 shows a nodule with a functional core of fasciculata tissue. Figure B 2 is a diagram of the nodule seen in figure 9. It has developed a bulge toward the main body of the gland, and the capsule has become thin at this point of closest approximation. In figure B 3, this separating membrane has been pierced. The arcuata tissue of the nodule begins to move into the general cortex. In the next diagram (B 4), more of the zona arcuata of the nodule has been drawn inward, and since this tissue is first to be invaginated and is coherent in itself, the distal surface of the nodule has been denuded from its zona arcuata. With figure B 5 we have reached the stage documented in figure 11: The greater part of the arcuata tissue has been drawn into the gland. The outer two-thirds of the nodule consist of fasciculate tissue only. In figure B 6, all the arcuate tissue has been incorporated into the main body of the gland, the fasciculate tissue now follows into the depth. The phase of figure 10 is reached. It can be expected that subsequently, the remainder of this fasciculate tissue will be drawn into the gland.

The use of the phrase "drawn into the gland" must now be motivated. The observer has the impression that an attraction exists between masses of cortical tissue, so that the nodules are actually drawn into the general cortex. This impression becomes stronger after comparison with the incorporation of nodules in the goat, which evidently is achieved by pressure exerted by the elasticity of the capsule, as will be shown. If the bridges shown in figures 10 and 11 do not exist by mere accident, a kind of tropism may be sought as an explanation for them.

The only alternative explanation which can be found for a picture like this would be eruption of zona arcuata tissue. This possibility can, however, be excluded for two reasons: (1) Since it has been established that the inner layers of the cortex have to be replaced from the outer layers, it would be quite paradoxical to assume that a centrifugal movement of cortical tissue should take place. (2) The perforated, funnel-shaped portion of the capsule shown in figure 11 clearly points inward. In case of an outward movement, this funnel would act as a valve, *i.e.*, it would be closed and thus prevent streaming out of tissue. If

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PLATE 2

Fig. 7—Newly formed groups of cortical cells in the capsule of the adrenal gland of a horse in various stages of development. The stages shown in this figure lead to the formation of nodules such as those shown in figures 9 and 11.

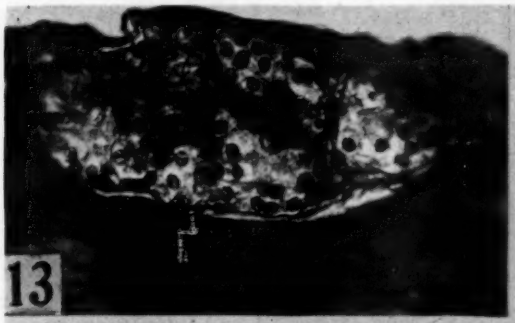
Fig. 8—Newly formed cortical laminae (horse) being pushed from the right to the left and folded by mutual pressure. The laminae thus formed are being incorporated directly into the cortex. Younger cell groups at the right, older laminae at the left.

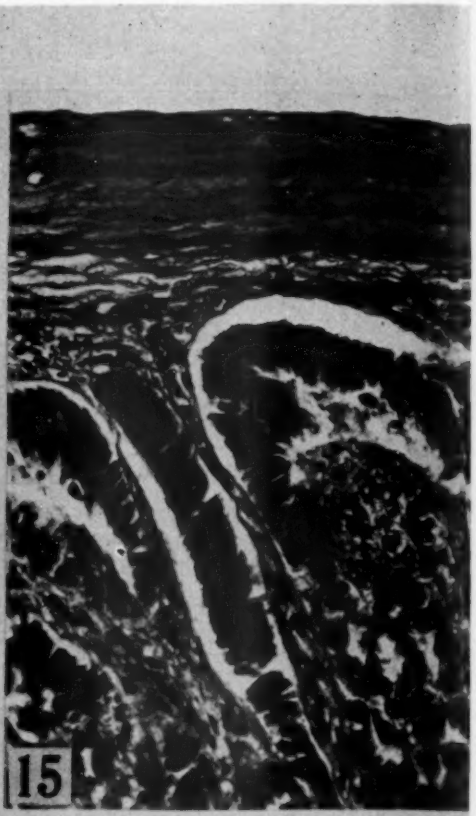
Fig. 9—A cortical nodule in the capsule of the adrenal gland of a horse. The nodule consists of an outer layer of laminae arcuata and a core of zona fasciculata. Contact with the general cortex is about to be established at the lower right. (Corresponds to fig. B 2.)

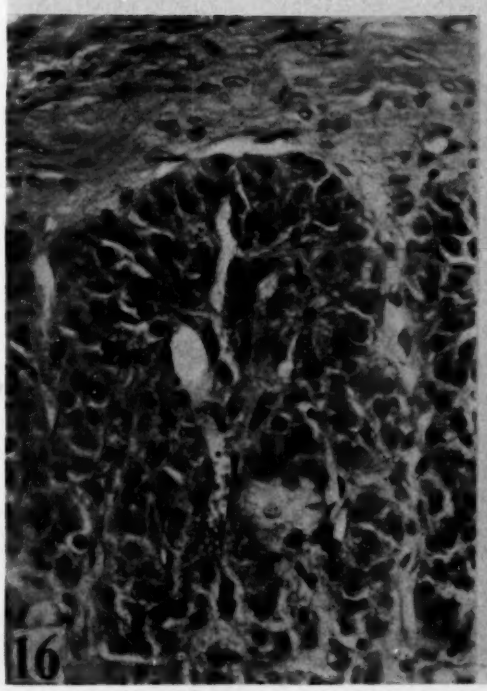
Fig. 10—A mass of cortical tissue (horse) has been formed at the very periphery of the capsule. A bridge of cortical tissue connecting this mass with the main cortex has been formed. Small foci of cortical cell formation are also seen in this picture, in the capsule. (Corresponds to fig. B 6.)

Fig. 11—A cortical nodule in the capsule of the adrenal gland of a horse, consisting of zona arcuata proximally and zona fasciculata distally. A funnel-like opening has been established in the capsule through which the nodule is drawn into the main cortex. (Corresponds to fig. B 5. At the left, part of nodule of fig. 9.)

Fig. 12—Horizontal section through the zona fasciculata of the adrenal gland of the horse. The section shows that this zone appears as a continuous parenchyma (not as cords), perforated by cylindrical blood vessels.







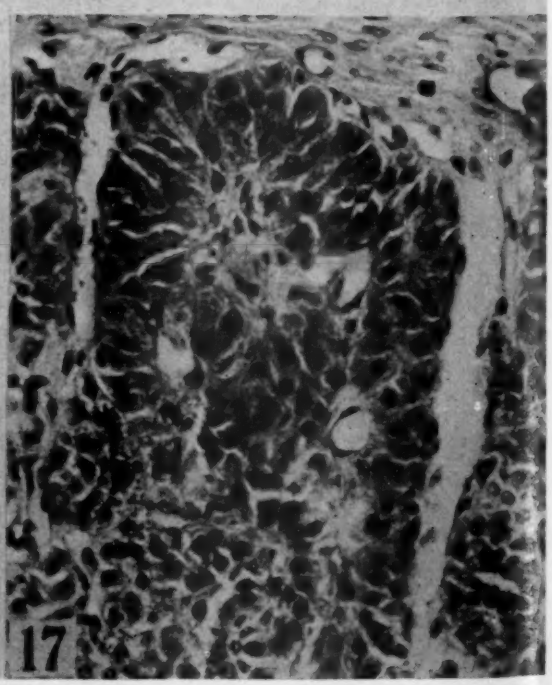


Plate 3

an outward movement existed at this place, the edges of the funnel would have to point outward. Thus, the only remaining explanation for this picture is inward movement. Pressure by an outside force (elasticity of the capsule) must also be excluded, for the part of the capsule on the parietal side of the nodule is extremely thin.

2) *Cell Groups of Laminary Character*—Figure 8 shows a quite different arrangement of cortical cells in the capsule. At *a* we find three small groups of very small cells. To the left of them is seen a horizontally located, plane, but typical, lamina. Further to the left, and in a straight line with the four cell groups described, follows a folded, arcuata lamina (at *b*). Immediately adjoining are further arcuata laminae. At the left margin of figure 8, this straight row of intracapsular cortical cell groups is connected with the arcuate zone of the main gland. We can describe this row of cortical material as intracapsular. Indeed, we find above it and below it thick layers of fibrous tissue, except at the left, *i.e.*, at the point of contact with the gland proper. A second row of the same character is found in a deeper layer of the capsule, also connected, at the left, with the main cortex.

DISCUSSION. — Whether cell groups newly formed in the capsule develop into nodules before their incorporation into the gland or are immediately incorporated into the zona arcuata depends, perhaps, on the shape and direction of the tissue spaces into which they can squeeze themselves. If solid tissue prevents extension in one direction, a growing lamina must necessarily fold itself and will form a nodule. If the lamina develops in a flat tissue space which is directed toward the cortex, the lamina will push itself, without bending, between the existing arched membranes, and a flat, vertical lamella will be the result.

Now, it can occur (*see* fig. 8), that cell group after cell group is formed in rapid succession at the end of a flat tissue space. The older cell groups will then be pushed forward by the newly developing ones. It can be assumed that the three cell groups in figure 8 at *a* are young stages of a developmental process. The groups to the left of them appear to be more advanced. All these young laminae are growing and attempting to expand. Since their growth is faster than they can gain entrance into the zona arcuata, they are thrown into folds, as is seen in figure 8 at *b*.

This seems to be the typical method by which the complicated shapes of the arcuate lamellae come into existence.

Figure 8 shows two such developmental rows, overlapping. This explains why we find, occasionally, two layers of arched membranes one on top of another (*see* fig. 6[5]).

3) *Direct formation of secretory cells.*—As has been shown[5] (*see* fig. 7b[5]), some of the newly formed cell groups do not develop to tall columnar epithelial form, but they become isodiametrically polygonal (they do not form membranes) and assume immediately the character of the zona fasciculata. Thus, small groups of secretory cortical cells are occasionally found in the capsule.

Among the three horse adrenals examined, only one showed evidence of new cortical tissue being formed in the capsule. Gruenwald and Konikov[8] already noticed that in those mammals in which the zona glomerulosa is well developed and is made up of large cell groups, cell replacement from the cortex occurs infrequently. Gruenwald[7] says: "On the assumption that the zone is a reserve store of cortical cells, this may be interpreted as indicating that organs with a large store need supplies from the outside less frequently than others."

During a period when cell replacement occurs, however, it seems to be very intense. In the individual horse adrenal, in which cell replacement occurs, it is very rich. Figure 7 shows, in a very small area, eleven nuclei of formation of new cortical tissue. Figure 8 shows at least ten such foci in a smaller area.

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PLATE 3

Fig. 13—A group of medullary cells in the capsule of the adrenal gland of a pig.

Fig. 14—Capsule and distal part of the zona arcuata of the adrenal gland of a pig. The capsule is divided into an outer, fibrous portion and an inner, cellular portion ("blastema" of Bachmann). New groups of cortical cells are being formed in the blastema which appear to push their way obliquely into the cortex.

Fig. 15—Vertical section of the zona arcuata of a pig.

Fig. 16 to 17—Glomeruli from the zona glomerulosa of the adrenal gland of a goat. Sinusoids are seen penetrating from below into the glomerulus of figure 16, dividing its lower portion into cords. Figure 17 shows a glomerulus surrounded by sinusoids, and three sinusoids in its interior, obliquely directed.

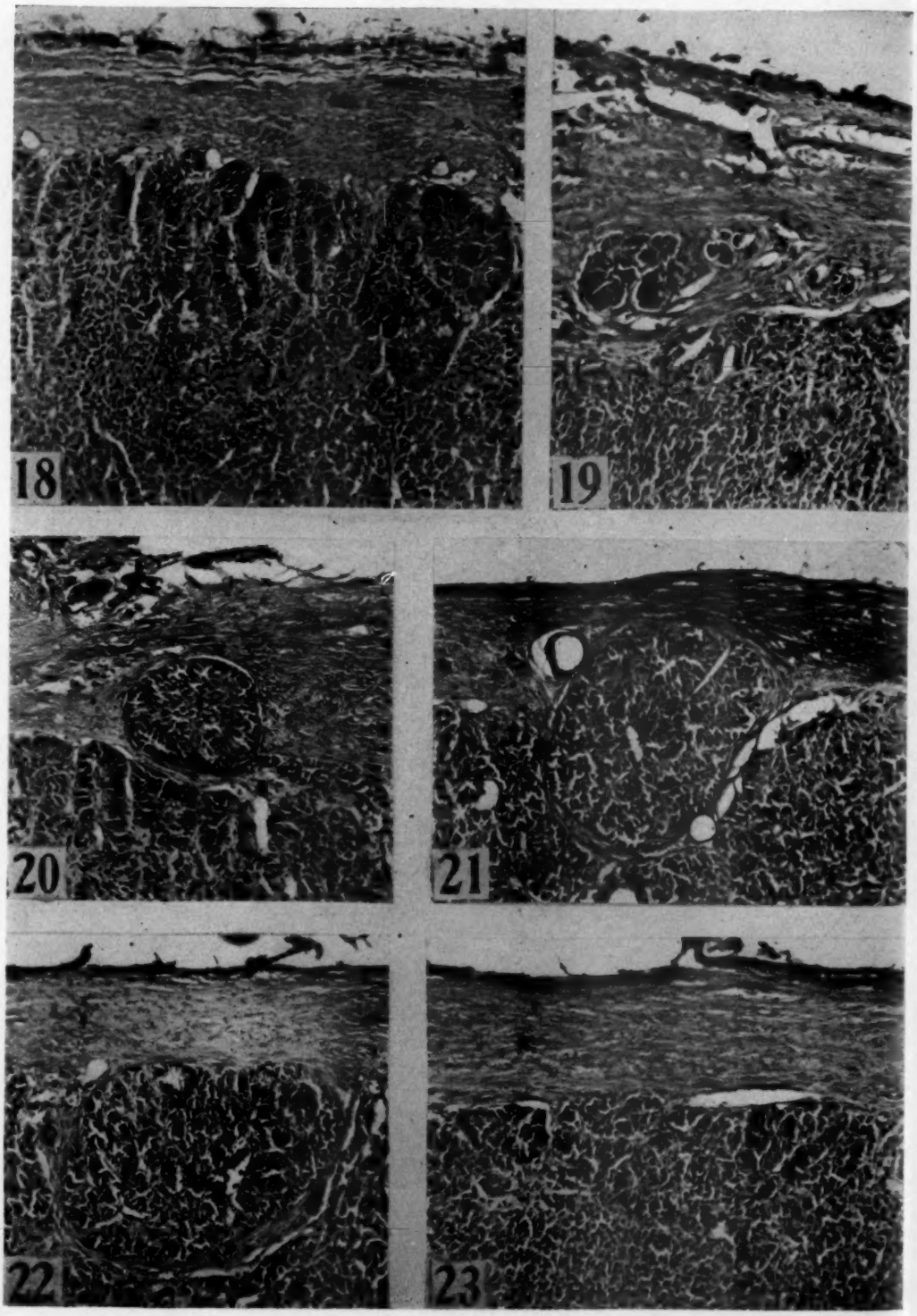

Plate 4

C) THE ZONA FASCICULATA

The zona fasciculata of the horse is not composed of cords, but has in routine sections the appearance of a continuous parenchyma, pervaded by parallel cylindrical blood spaces. Through the presence of these, the zona fasciculata of the horse has, in vertical sections, the same appearance as that of any other mammal. But if tangential sections are examined (fig. 12), we see the continuity of the tissue. This is perforated by the circular cross sections of the cylindrical, parallel sinusoids.

Thus it is seen that this region of the adrenal cortex of the horse should not be called zona fasciculata, but rather zona pseudoparenchymatosa. The prefix pseudo is used, because Gruenwald[7] has shown that the cells of this zone are separated by argyrophil fibers.

D) MEDULLARY CELL GROUPS IN THE CAPSULE

Groups of medullary cells are frequently found in the capsule. No picture of this condition is added here, because these cell groups are so large that their location in the capsule can only be demonstrated in a very low power picture, while a high magnification would be required to prove their medullary character. Therefore, reference is made to figure 13, which represents a small group of medullary cells in the capsule of the adrenal gland of a pig. Identification of these cells as medullary was made by comparison of size, shape, and protoplasmic structure with the cells found in the medulla of the same gland. Flint[6] observed medullary cell groups in the zona glomerulosa of the pig, dog, and cat.

II) THE PIG

The pig too possesses a typical zona arcuata (fig. 15). But the arched membranes are less extended and less folded than those of the horse. They give the impression of being more rigid, less pliable. The cells which compose them are lower than those of the horse. They are arranged in several layers (fig. 14 and 15).

The capsule consists of two distinct layers—an outer, fibrous layer and an inner, cellular layer (fig. 14 and 15). It is in the inner cellular layer that the formation of new cortical tissue occurs. We have here a condition similar to that described by Bachmann[1] for kittens; he called the inner cellular layer "blastema."

Figure 14 shows three young cortical cell groups in various stages of development. The picture conveys the impression that older cell groups are pushed obliquely into the cortex by the younger, expanding cell groups. This method of cell replacement is similar to the second method occurring in the horse (*see* fig. 8). As has been mentioned, groups of medullary cells are found in the capsule (fig. 13).

III) THE GOAT

A) STRUCTURE OF THE ZONA GLOMERULOSA

The outer layer of the adrenal cortex of the goat appears intermediate between a zona glomerulosa and a zona arcuata (fig. 18). It consists of rather large cell groups, some of which appear solid (as the glomerulus shown at the right of fig. 18), while others resemble more the arches of the horse (center fig. 18). Figures 16 and 17 show two typical glomeruli of the goat, highly magnified. They are large, solid, groups of cells, pierced by blood sinusoids. If a fairly large sinusoid is located in the center of the glomerulus, the resemblance to an equine arcus is great. But the resemblance is only superficial. For the genesis of the caprine glomeruli is entirely different from that of the folded, arched laminae of the horse. In figure 16 we see four sinusoids penetrating from below into a glomerulus, splitting it up

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PLATE 4

Vertical sections of the capsule and cortex of the adrenal gland of the goat.

Fig. 18—Typical section of capsule and adrenal cortex of goat.

Fig. 19—Groups of new cortical cells being formed in the capsule.

Fig. 20—A young nodule in the capsule.

Fig. 21—An older nodule which, growing, has stretched the capsule and pushed it outward. The nodule is being pushed downward into the cortex, probably by a tendency of the capsule to regain its former structure.

Fig. 22—A nodule which has been pushed downward into the cortex. Its distal boundary has been flattened. The original structure of the capsule is restored.

Fig. 23—Last stage of incorporation of a nodule into the cortex. The nodule has been flattened more than in the preceding picture; the fibrous tissue which had separated it from the cortex has disappeared. Its boundary toward the cortex has become indistinct. The underlying glomeruli have been transformed into zona fasciculata tissue. It is now a part of the zona glomerulosa.

into cords which are continuous with the cords of the zona fasciculata. Figure 17 shows more distinctly the external vascularization of a glomerulus. It also shows three internal sinusoids, obliquely sectioned.

B) FORMATION OF NEW CORTICAL TISSUE

Figures 19 to 23 represent successive stages of appositional growth of the adrenal cortex of a goat. It appears that nodules are formed in the cortex from undifferentiated cells. Figure 19 shows three nodules in the capsule which appear to be very young, for they consist of a small number of little cells. The smallest and probably youngest is seen at the right, a slightly larger one in the center, and a still larger one at the left. These three nodules consist of irregular cell groups.

In figure 20, which appears to present a later stage, the nodule has become rounded and solid. A larger nodule (fig. 21) is pervaded by blood vessels. These split the solid mass into glomeruli. The growing nodule is being pushed into the cortex. Evidently, this is accomplished in the following way: The growing nodule exerts pressure on the capsule and causes a transverse compression and longitudinal stretching of its fibers and an outward bulge. The fibers of the capsule are obviously pushed upward and pressed together so that the fibrous tissue of the capsule is, above the nodule, denser (and thus stains deeper) than at other places. It is probably the elasticity of this compressed part of the capsule which tends to restore its original structure, and thus presses the nodule downward and flattens out its parietal surface. Such a nodule (fig. 22) is situated entirely at the level of the zona glomerulosa, but it is still separated from the cortex by a thin, fibrous membrane, a part of the capsule. At this time, the outer part of the capsule has regained its former structure. The connective tissue membrane which bounds the nodule at its proximal surface finally disappears (fig. 23).

In this latest stage of development, the nodule has become more flattened, and its boundaries have become indistinct. It resembles now a plano-convex lens. A connection with the zona fasciculata is now being established; the incorporation is almost completed

IV) CATTLE

A) STRUCTURE OF THE ZONA GLOMERULOSA

The bovine zona glomerulosa consists of elements of various size and shape (fig. 24). Among them are small, irregularly shaped cell groups; small, curved membranes; and bodies shaped like cups or hollow balls. The most characteristic glomeruli are large, hollow bodies which appear like collapsed balls (left, fig. 24). In tangential sections of the zona glomerulosa, these appear rosette-like. All these membranes, cups, and balls consist of simple cuboidal to low columnar epithelium.

B) FORMATION OF NEW CORTICAL TISSUE

Two methods of regeneration of the zona glomerulosa were found:

1) *Simple Proliferation.* — The simpler of these two methods gives rise to the smaller glomeruli. At A in figure 24, it can be seen that cells from the inner border of the capsule proliferate into the zona glomerulosa penetrating between its elements like a wedge. The deeper they come into the cortex, the more they enlarge and acquire finally, at B, the character of the cells of the zona glomerulosa.

2) *Nodule Formation.* — The second, more complicated method of cell replacement is through formation of nodules which consist wholly of zona glomerulosa tissue. The nodules are finally added to the cortex in a manner similar to that found in the goat.

The origin of the nodules themselves takes a very different course. Figure 25 shows a small, evidently young nodule. It consists of folded membranes made up of simple, low columnar and cuboidal epithelium. On the right side of this nodule we find a membrane, A, consisting of high cuboidal epithelium. This membrane tapers, as it approaches the periphery of the nodule at B. The cells are low cuboidal at this point. Further upward, the membrane tapers more and more, the cells being flat at this place, C. The membrane continues almost around the entire nodule to the point D. From this place on, its cells cannot be distinguished from mesenchyme cells.

There appears to be within this membrane a succession of developmental stages, the cells at A being the oldest, those at D being the youngest. This picture suggests that the membrane is expanding and pushing its way into the nodule, and that the growing membrane exerts a rotatory momentum upon the nodule. Whether a rotation is actually produced will depend, of course, on the friction which the nodule encounters in the surrounding tissue and also on the simultaneous action of other membranes invading the same nodule. At E and F we find another younger membrane invading the nodule in the same direction as the first membrane. Its rotatory momentum acts in the same direction. The other two membranes seen in the nodule are

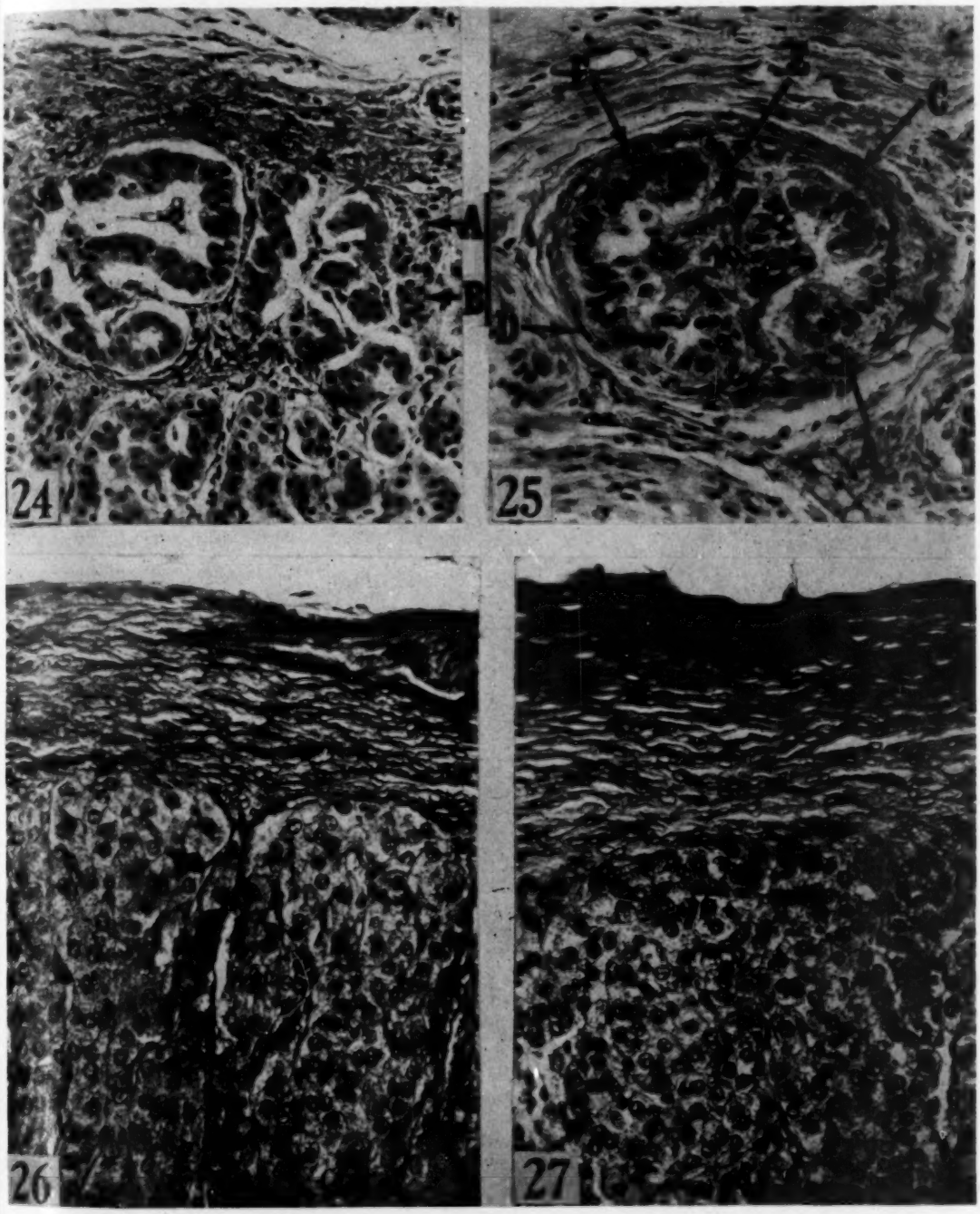

PLATE 5

Fig. 24—Zona glomerulosa of the adrenal gland of a calf. At the right, proliferation of capsular cells into the cortex and their transformation into cortical tissue.

Fig. 25—A cortical nodule in the capsule of the adrenal gland of a calf.

Fig. 26—A typical section of the outer part of the adrenal gland of a lamb. Note indistinctness of zona glomerulosa or arcuata.

Fig. 27—Fluent transformation of cells of the capsule into cortical tissue.

not sectioned properly to show the direction in which they invade the nodule. It may be that they are already fully incorporated in it. If they penetrate into the nodule in a direction different from or opposite to that of the other two membranes, they may counteract or complicate the rotatory movement which might result from the expansion of the first two membranes.

As the membranes push their way into the nodule, they are being folded, thus giving rise to the membranous glomeruli.

The nodule shown in figure 25 is still young and relatively small. The bovine adreno-cortical nodules grow to a much larger size before they are pressed into the general cortex. They maintain the character of zona glomerulosa as long as they are located in the capsule.

The complicated genesis of the nodules, as outlined, accounts for the complicated shape of the large glomeruli (left, fig. 24). Smaller glomeruli (center and lower third, fig. 24) may be fragments of large ones or may have arisen by immediate proliferation of capsular cells into the cortex (fig. 24, A, B).

V) SHEEP

A) STRUCTURE OF THE ADRENAL CORTEX

The most remarkable feature of the adrenal cortex of the sheep is the indistinctness or even absence of a zona glomerulosa or arcuata. Flint states that "the zona glomerulosa shows very little differentiation from the remainder of the cortex." At certain places there might be a slight demarcation of a zona glomerulosa (fig. 26), but at other places (fig. 27), the zona fasciculata begins immediately beneath the capsule. That means that new cells and cell groups acquire the character and arrangement of the zona fasciculata as soon as they are incorporated in the cortex.

The capsule is occasionally clearly divided into an outer, fibrous and an inner, cellular layer (fig. 29). Sometimes the transition from the fibrous to the cellular layer is gradual (fig. 27). In other cases or at other places, the capsule is entirely fibrous (fig. 31) or entirely cellular (fig. 30). The character of the capsule of one gland frequently changes from place to place. Figures 30 and 31, for example, are taken from the same gland but show opposite character.

B) FORMATION OF NEW CORTICAL TISSUE

Cell replacement for the cortex occurs in two fundamentally different ways:

1) *Fluent Transition.*—The first and simpler method is by gradual, fluent transformation of the capsule into the zona fasciculata (fig. 27). The cells of the capsule enlarge gradually and are gradually transformed into cortical cells. There is no sharp boundary between capsule and zona fasciculata at the places where this kind of cell replacement occurs (fig. 27).

This method of cell replacement has been described by Bachmann[1] for the cat. While this kind of transition takes place over large surfaces of the gland of sheep, it is not found everywhere at the same time; but there are places (fig. 26) where the boundary between the capsule and the cortex is sharp and distinct. It seems from this that periods of intense cortical formation (fig. 27) interchange with periods of rest (fig. 26) from place to place.

At other places (fig. 28), one can observe that masses of newly formed cortical tissue move horizontally along between the capsule and the cortex, to a point from which they move obliquely downward to be incorporated in the cortex.

2) *Formation of Nodules.*—The other method of cell replacement is by the formation of nodules and their later incorporation.

Two kinds of nodules have been found. In figure 29 is a nodule which seems to be floating in a space filled with liquid. This space is lined by flat cells of endothelial appearance. The nodule itself has very much the appearance of the corona radiata around a mammalian oöcyte, the peripheral cells being fringed.

Another type (fig. 31) is characterized by the nodules within the capsule being surrounded by individual capsules of dense fibrous tissue. The nodules themselves are solid, parenchymatous cell masses. The capsule is immediately adjacent to the outer cells of the nodule.

An early stage of development of this type of nodule is seen in figure 30. Concentric arrangement of fibroblasts and fibers around a small mass of new cortical cells marks the beginning of the formation of an encapsulated nodule.

There seems to be but one type of cell replacement going on in any one ovine adrenal gland at one time. Thus, in lamb 1, only the fluent transition from capsule to zona fasciculata was found; in lamb 2, only fringed nodules floating in a liquid-filled space were seen; in lamb 3, only nodules with fibrous capsules were observed. This is in marked contrast to other animals investigated, where we frequently can find several types of growth in a single gland at the same time.

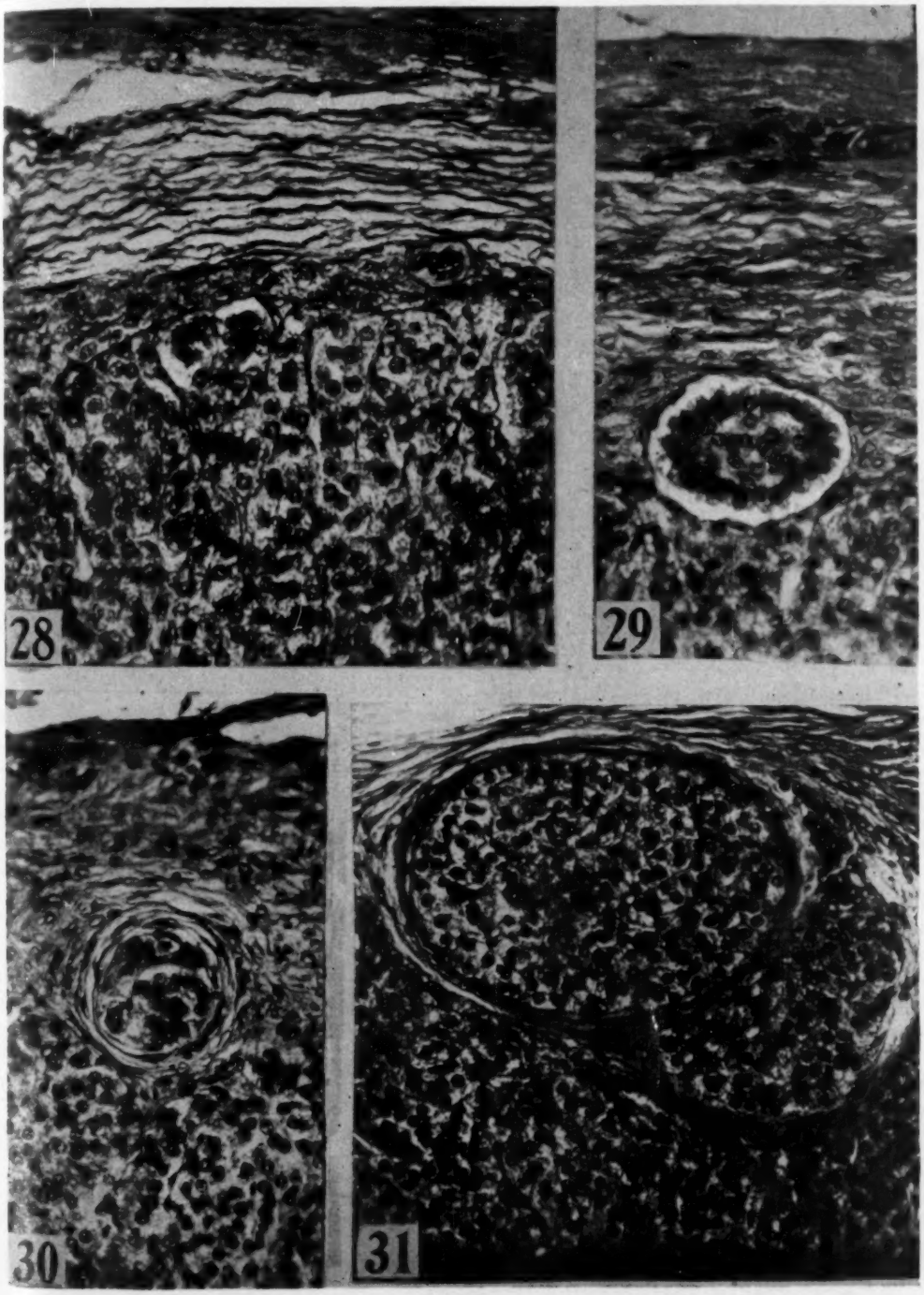

PLATE 6

Fig. 28—Capsular cells being incorporated in an oblique direction into the adrenal cortex of a lamb.
Fig. 29—A nodule of young cortical tissue in the capsule of the adrenal gland of a lamb. Nodule of the fringed type, floating in a liquid-filled space. Capsule divided into outer fibrous and inner cellular zone.
Fig. 30—Formation of an encapsulated nodule in the adrenal capsule of a lamb.
Fig. 31—Two encapsulated cortical nodules being pushed from the capsule into the cortex (same lamb as fig. 30).

CONCLUSION

The methods of cell replacement for the adrenal cortex are most varied. In the present study, several types have been shown which were not included in Gruenwald and Konikov's classification.[8] Since we have seen up to three different modes in a single species, it can be expected that more methods of cell replacement will be found, not only as more mammalian species are investigated, but also as more specimens of the same species are studied.

SUMMARY

The structure of the adrenal cortex of the horse, the pig, the ox, the goat, and the sheep is described as follows:

The horse possesses a typical zona arcuata consisting of folded laminae which are made up of pseudostratified epithelium.

The zona arcuata of the pig is similar, but the arched laminae are less extended, less folded, and consist of several layers of low columnar cells.

The goat has a zona glomerulosa. The glomeruli are large and internally vascularized.

The ox possesses large, hollow glomeruli, lined by simple, cuboidal epithelium, and small glomeruli having the shape of curved membranes.

The sheep does not possess a distinct zona glomerulosa or zona arcuata.

The statements by Zwemer, Wotton and Norkus,[15] Bachmann.[1] and Gruenwald and Konikov[8] that the cells of the adrenal cortex are replaced by morphogenesis of undifferentiated cells of the capsule, are confirmed for the five species. The following survey includes the modes of adrenocortical growth previously described, as well as the newly found methods:

1) The cells of the capsule enlarge along a broad front, move downward and are gradually transformed into cortical cells and incorporated into the cortex (cat, Bachmann;[1] sheep, fig. 27).

2) A few cells from the inner boundary of the capsule proliferate locally, penetrate wedgelike between the existing glomeruli, and acquire the character of cortical cells (cattle, fig. 24, A, B).

3) Broad plates of new cortical tissue are formed in the capsule and fuse with the cortex (guinea pig, Gruenwald and Konikov[8]).

4) Parenchymatous nodules of cortical tissue are formed in the capsule by local accumulation of cells. These are pushed downward by an unknown force (sheep, fig. 29, 31; guinea pig, Gruenwald and Konikov;[8] man, Gruenwald[7]).

5) Parenchymatous nodules are formed in the capsule; they are subdivided into glomeruli by invading sinusoids. The nodules are pressed downward by the elasticity of the capsule (goat, fig. 20 to 23).

6) Glomerulous nodules are formed by the expansion and folding of growing membranes composed of simple cuboidal epithelium. These are pushed into the cortex by an unknown force (cattle, fig. 25).

7) Nodules are formed by the development and expansion of laminae composed of columnar cells which are folded for lack of space. These nodules grow and form a zona fasciculata in their interior. They are drawn into the cortex through funnel-like openings in the capsule, in response to an attractive force (horse, fig. 7, 9, 11, and fig. B).

8) Laminae of columnar cells are formed in the capsule and pushed immediately between existing arcs of the zona arcuata (horse, left fig. 8).

9) Successive laminae of columnar cells are formed in the capsule. The younger ones press the older ones together in a horizontal direction and cause their folding into arched laminae of the zona arcuata (horse, fig. 8; pig, fig. 14).

It should be noted that methods 8 and 9, as well as the initial phase of method 7, are variations of the same process, modified only by the shape of available tissue spaces.

10) Cell cords are formed in the capsule. They are joined, end to end, to cords of the cortex (rhesus monkey, Gruenwald and Konikov[8]).

References

[1]Bachmann, R.: Zur Frage der Zona germinativa der Nebennierenrinde. Klin. Wchnschr., *18*, (1939): 783-784.

[2]Bennett, H. Stanley: The Life History and Secretion of the Cells of the Adrenal Cortex of the Cat. Am. J. Anat., *67*, (1940): 151-227.

[3]Blumenthal, Herman T.: The Mitotic Count in the Adrenal Cortex of Normal Guinea Pigs. Endocrinology, *27*, (1940): 477-480.

[4]Dagonet, J.: Beitrage zur pathologisthen Anatomie der Nebennierenrinde des Menschen. Ztschr. f. Heilk., *6*, (1885): 1-25.

[5]Elias, Hans: Comparative Histology of Domestic Animals. III. Endocrine Glands 1. The Adrenal Bodies. Middlesex Vet., *5*, (1945): 12-16, 36-38.

[6]Flint, Joseph Marshall: The Blood Vessels, Angiogenesis, Organogenesis, Reticulum, and Histology of the Adrenal. Contr. to the Sci. of Medicine, Johns Hopkins University Press, (1900): 153-230.

[7]Gruenwald, Peter: Common Traits in Development and Structure of the Organs Originating from the Coelomic Wall. J. of Morphol., *70*, (1942): 353-387.

[8]Gruenwald, Peter, and Konikov, William M.: Cell Replacement and Its Relation to the Zona Glomerulosa in the Adrenal Cortex of Mammals. Anat. Rec., *89*, (1944): 1-21.

[9]Hoerr,, Normand: The Cells of the Suprarenal Cortex of the Guinea Pig. Their Reaction to Injury and Their Replacement. Am. J. Anat., *48*, (1931): 139-197.

[10]Lewis, R. W., and Pappenheimer, A. M.: A Study of the Involutional Changes which Occur in the Adrenal Cortex During Infancy. J. Med. Res., *34*, (1916): 81-94.

[11]Mulon, P.: Excrétion des capsule surrénales du cobaye dans les vaisseaux sanguins. Compt. Rend. Soc. de Biol., *54*, (1902): 1540-1542.

[12]Mulon, P.: Divisions nucléaires et rôle germinatif de la couche glomérulaire des capsules surrénales du cobaye. Compt. Rend. Soc. de Biol., *55*, (1903): 592-595.

[13]Swinyard, C. A.: Growth of the Human Suprarenal Glands. Anat. Rec., *87*, (1943): 141-150.

[14]Williams, Roy G.: Studies of Adrenal Cortex: Regeneration of the Transplanted Gland and the Vital Quality of Autogenous Grafts. Am. J. Anat., *81*, (1947): 199-232.

[15]Zwemer, R. L., Wotton, R. M., and Norkus, M. G.: A. Study of Corticoadrenal Cells. Anat. Rec., *72*, (1938): 249-263.

[16]Gruenwald, Peter: Embryonic and Postnatal Development of the Adrenal Cortex, Particularly the Zona Glomerulosa and Accessory Nodules. Anat. Rec., *95*, 1946): 371-421.

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AMERICAN VETERINARY MEDICAL ASSOCIATION
600 So. Michigan Avenue, Chicago 5, Illinois

The Viability of Dried Viruses of Avian Origin

F. R. BEAUDETTE, D.V.M., BARBARA RHODES MILLER, B.S.,
J. A. BIVINS, D.V.M., and C. B. HUDSON, B.S., M.S.

New Brunswick, New Jersey

MANY viruses of avian origin have been propagated in embryonating eggs in this laboratory for several years. Some strains undergo passage only infrequently and for the most part are maintained in a dried state under vacuum. Samples of those undergoing regular passage are occasionally dried and stored as a backlog against loss of the passage strain. Thus, there has been built up a store of viruses that have been held in a dried state for various periods. Some of these have been tested for activity from time to time, and recently several were suspended in nutrient broth and inoculated into eggs. Since several of these were found to be active after being held in a dried state for protracted periods, it was thought that this information might be of value to others.

Passage strains were held in the freezing chamber of a refrigerator until 1942 and thereafter in a freezer at –20C. Samples to be dried were processed a few days to approximately a month after harvesting and meanwhile were held in a frozen state. Unfortunately, the actual date of drying was not recorded in several cases. Samples to be dried were usually ground with sterile sand, suspended in a minimal quantity of broth, and then distributed by means of a pipette to an appropriate number of tubes, which were frozen in a slanted position. The samples were placed in a desiccator over phosphoric anhydride and dried under vacuum from the frozen state. In a lightly charged small desiccator, drying was usually completed in two days. Dried samples were thereafter stored over anhydride in a large evacuated desiccator in the refrigerator.

Paper of the Journal Series, New Jersey Agricultural Experiment Station, Rutgers University, Department of Poultry Pathology.

Research specialist (Beaudette) and assistant research specialist (Hudson) in poultry pathology; research workers (Miller—assumed duties June 1, 1946); Bivins—assumed duties Dec. 1, 1946).

VIRUSES TESTED

Laryngotracheitis.—Two strains of this virus were tested. One strain was first inoculated into embryonated eggs on Dec. 12, 1935, and since then has been passaged exclusively in chicken eggs. To date (April 29, 1947) it has undergone 163 passages. We have already reported on the viability of vaccine made from this strain.[1]

The second strain (Kurcheck) was first injected into eggs on Sept. 22, 1943, as a Berkefeld V filtrate of respiratory exudate collected from a field case presented for diagnosis on September 12. Unlike most laryngotracheitis strains, this one has never produced extensive lesions; in fact, with passage lesions seemed progressively less extensive. By the ninth passage further inoculations were discontinued, and another series was started with a fifth passage membrane that had been frozen for 162 days. Growth, as in the first series, was again progressively less in subsequent passages, which were discontinued with the twelfth because of the absence of grossly visible lesions.

The results of the tests on viability of

TABLE I—Viability of Two Strains of Egg-Propagated Larnygotracheitis Virus Held in a Dried State

No.	Passage	Harvest date	Dried	Days held	Tested	Result*
			First Strain			
L314	21	7/17/36		990	4/ 3/39	I
L658	51	2/26/38		3,359	5/ 9/47	I
L946	68	1/12/39	1/19/39	3,039	5/ 9/47	I
L960	69	2/16/39		3,004	5/ 9/47	I
L1017	73	4/12/39		2,949	5/ 9/47	I
Pool	90	2/21/40		311	12/28/40	I
L1746	92	5/ 3/40	5/ 7/40	2,562	5/ 9/47	I
L3443	141	5/ 9/44	5/13/44	1,095	5/ 9/47	I
L3633	149	2/28/45	3/30/45	806	5/15/47	I
L3793	156	11/21/45	1/30/46	541	5/16/47	I
L3830	157	2/ 5/46	3/11/46	465	5/16/47	I
			Kurcheck Strain			
L32	5	5/ 9/44	6/16-17/44	1,095	5/ 9/47	N
L36	6	6/21/44	7/12/44	1,052	5/ 9/47	N
L64	6	10/24/44	10/27/44	927	5/ 9/47	I

* I= Infective. N = Noninfective.

the several samples of dried laryngotracheitis virus are given in table 1. The table shows that 11 samples of the first strain from the twenty-first to the 157th passages were tested. All samples were active even to a holding period of over nine

TABLE 2—Viability of Chicken and Duck Egg-Propagated Fowlpox Virus in a Dried State

No.	Passage	Harvest date	Dried	Days held	Tested	Result*
Fowlpox in Chicken Eggs						
F123	22	7/ 6/37		3,598	5/13/47	I
F191	28	2/26/38		3,363	5/13/47	I
FH117	37	12/13/38	1/19/39	3,073	5/13/47	I
FH384	53	5/ 5/40		2,564	5/13/47	N
FH385	53	5/ 5/40		2,564	5/13/47	I
Fowlpox in Duck Eggs (Series 1)						
FD1	38	6/13/38		50	8/ 2/38	I
FD2	38	6/13/38		105	9/26/38	I
FD59	42	2/ 1/39		1,928	5/13/44	I
Fowlpox in Duck Eggs (Series 2)						
FD71	43	2/ 1/39		453	4/29/40	I
FD120	44	5/ 5/40		684	3/20/42	I
FD121	44	5/ 5/40		851	9/ 3/42	D
FD107-14	44	5/ 5/40		881	10/ 3/42	I
FD147	45	10/ 9/42		582	5/13/44	I
FD147	45	10/ 9/42		1,687	5/23/47	I
FD187	48	6/26/44	7/12/44	310	5/ 2/45	I

* I = Infective. N = Noninfective. D = Doubtful.

years (3,359 days). In the Kurcheck strain, however, two samples were inactive after being held about three years. One sample held 927 days was active. Incidentally, the disparity in harvest dates between the two sixth passage samples tested resulted from the starting of a second series referred to above.

No attempt has been made to determine the viability of samples held in a frozen state. In routine serial passages, however, one strain was active after 111 days. This was a 161st passage harvested on Oct. 7, 1946, and tested on Jan. 26, 1947. The Kurcheck strain, as already mentioned, was active after being held 162 days in a frozen state.

Fowlpox.—The first passage of this virus was initiated in eggs on March 8, 1936, by inoculation of a bacteriologically sterile suspension of scrapings from the flesh side of skin, opposite an area inoculated by the feather-follicle method. At the present time, this strain has undergone 109 serial passages in eggs.

This strain of fowlpox was also transferred to duck eggs on May 3, 1937, after 20 passages in chicken eggs and, to date, has been carried through 25 passages in duck eggs, that is, for a total of 45 passages in eggs.

A second series was started in duck eggs on Oct. 11, 1938, after 39 passages in chicken eggs. This series has undergone a total of 52 passages.

TABLE 3—Viability of Chicken and Duck Egg-Propagated Pigeon Pox Virus Held in a Dried State

No.	Passage	Harvest date	Dried	Days held	Tested	Result*
First Strain in Chicken Eggs						
P278	21	7/ 6/37		3,605	5/20/47	I
PH190	47	1/12/39	1/19/39	3,050	5/20/47	I
PH191	47	1/12/39	1/19/39	3,050	5/20/47	I
PH424	61	4/ 4/40		2,602	5/20/47	N
PH957	85	9/29/42	10/20/42	1,694	5/20/47	I
PH1297	106	10/18/44	10/27/44	944	5/20/47	I
Pigeon Pox in Duck Eggs						
PD5	42	6/13/38		50	8/ 2/38	I
PD60-62	45	2/ 1/39		436	4/12/40	I
PD131	47	4/17/39		361	4/12/40	I
PD221	50	5/ 6/40		343	4/14/41	I
PD221	50	5/ 6/40		357	4/28/41	I
PD251	51	4/21/41	5/13/41	530	10/ 3/42	I
PD271	52	6/16/41	6/17/41	444	9/ 3/42	I
PD287	52	6/16/41	6/17/41	277	3/20/42	I
PD432	52	10/ 9/42		533	3/25/44	I
PD426	54	10/ 9/42		159	3/17/43	I
PD646	55	4/28/44	5/13/44	369	5/ 2/45	I
PD654	56	5/19/44	6/16/44	348	5/ 2/45	I
PD654	56	5/19/44	6/16/44	1,099	5/23/47	I
Pigeon Pox Strain in Chicken Eggs						
PP103	10	5/ 5/40		2,574	5/23/47	N

* I = Infective. N = Noninfective.

The results of the tests on the viability of fowlpox samples are recorded in table 2. The table shows that fowlpox virus propagated in chicken eggs retained its activity after being held for 3,598 days, or approximately ten years. One of the two samples held only 2,564 days gave negative results which cannot be explained except that FH 384 was from a dead embryo and FH 385 from a live one.

Propagation of fowlpox virus in duck eggs seemingly did not greatly affect the viability of the virus in a dried state, one series retaining activity after 1,928 days, and the other after 1,687 days.

Glycerinated fowlpox virus held in the freezing compartment was active after 143 days. This was a thirty-fourth passage harvested on June 25, 1938, and tested Nov.

15, 1938. A ninety-seventh passage membrane harvested Aug. 15, 1945, was active when tested Dec. 29, 1945, 136 days later. By no means are these likely to be end points; in fact, a thirty-ninth passage in duck eggs harvested on May 8, 1945, was active after being held in a frozen state to July 12, 1946, or 430 days.

TABLE 4—Viability of Egg-Propagated Canary and Turkey Pox Viruses Held in a Dried State

No.	Passage	Harvest date	Dried	Days held	Tested	Result*
			CP Strain Canary Pox			
CP202	21	5/ 5/40		2,571	5/20/47	I
CP1020	54	5/ 9/44		1,106	5/20/47	I
			S Strain Canary Pox			
S66	12	5/ 5/40		2,571	5/20/47	I
			Turkey Pox			
T23	4	5/ 5/40		1,279	11/ 5/43	D
T24	4	5/ 5/40		1,468	5/12/44	I
T196	5	5/18/44		1,093	5/16/47	I
T107	19	3/16/43		423	5/12/44	I

* I = Infective. D = Doubtful.

Pigeon Pox.—The isolation and propagation of one strain of pigeon virus has already been described.[2] The strain has now undergone 120 passages.

Six samples of the virus propagated in chicken eggs were tested for viability, as shown in table 3. The passages ranged from the twenty-first to the 106th. These were tested after being held in a dried state for 944 to 3,605 days, and with one exception (held 2,602 days) all were active. Unfortunately, the length of time this material was held in a frozen state before being dried is not known.

The same virus, cultivated in duck eggs after an initial 18 passages in chicken eggs, has undergone a total of 61 passages. Table 3 shows that samples from the forty-second to the fifty-sixth passages were tested after being held in a dried state from fifty to 1,099 days, and all were found to be active.

The longest holding period in a frozen state was a 109th passage harvested April 17, 1945, and tested Aug. 25, 1945, 130 days later. A fifty-seventh passage duck egg strain harvested May 21, 1945, and frozen was inoculated July 12, 1946, or 417 days later. Both samples were active.

A second strain of pigeon pox virus (PP) was initiated Sept. 14, 1939, by inoculating eggs with a sterile suspension of skin-scrapings as already described. This strain was never carried beyond the twentieth passage. A tenth passage, after being held in a dried state for 2,574 days, was found inactive.

Canary and Turkey Pox.—Two strains of canary pox virus have been propagated in chicken eggs. The first of these (CP) was initiated March 1, 1939, and the second (S) on March 7, 1939. Both were started with Berkefeld V filtrates. At present, the first has undergone 66 serial passages, and the second 48 passages. Table 4 shows that each strain has retained its activity in a dried state for at least 2,571 days.

In a frozen state, a sixty-third passage of the CP strain, harvested on July 10, 1946, was active when tested Nov. 12, 1946, or 125 days later. Under the same conditions, a forty-second passage of the S strain, harvested on Aug. 1, 1945, was active when tested 131 days later, on Dec. 10, 1945.

The turkey pox strain was inoculated into eggs as a Berkefeld V filtrate on Feb. 9, 1940, as already reported.[3] To date, the strain has undergone only 32 serial egg

TABLE 5—Viability of Egg-Propagated Bronchitis and Bronchitis-Like Viruses Held in a Dried State

No.	Passage	Harvest date	Dried	Days held	Tested	Result*
			B Strain			
B286	39	7/ 8/37		511	12/ 1/38	I
B284	39	6/ 6/37		513	12/ 1/38	I
B589	44	1/ 9/39	1/19/39	3,053	5/20/47	N
B590	44	1/ 9/39	1/19/39	3,053	5/20/47	N
B596	45	1/16/39	1/19/39	396	2/16/40	I
B641	46	2/19/40		2,647	5/20/47	N
B650	48	5/ 4/40		684	3/19/42	I
B347	49	2/ 3/38		301	12/ 1/38	N
B349	49	2/ 3/38		232	9/23/38	I
B701	52	10/12/42		1,684	5/23/47	N
B704	152	10/12/42		1,684	5/23/47	N
			Brandly Strain			
CAB9	3	1/30/39		3,021	5/ 9/47	I
CAB54	12	5/ 3/40		2,562	5/ 9/47	I
CAB55	12	5/ 3/40		2,562	5/ 9/47	I
			Williamson Strain			
WS77	14	12/ 9/38		3,077	5/13/47	I
WS79	14	12/ 9/38		3,077	5/13/47	I
WS129	23	5/ 3/40		2,566	5/13/47	I
WS131	23	5/ 3/40		2,566	5/13/47	I

* I = Infective. N = Noninfective.

passages. In a dried state, as shown in table 4, the virus has retained its activity for 1,468 days.

In a frozen state, a fifteenth passage membrane harvested July 4, 1946, was active when tested Nov. 22, 1946, or 141 days later.

Bronchitis and Bronchitis-like Viruses.—The bronchitis virus tested (B) was the original strain cultivated in eggs in this laboratory since April 7, 1936. In recent years two series, approximately 100 generations apart, have undergone passage simultaneously. This will account for a fifty-second and a 152nd passage of the same harvest date in table 5. To date, one series of the strain has undergone 171 passages, and the other only 73 passages. The table shows that 684 days is the longest time the virus has retained its activity in a dried state.

In a frozen state, fifty-ninth and 161st passages harvested on Feb. 3, 1944, and each tested July 12, 1944, or 160 days later, were shown to be active.

The Brandly strain (CAB) had its origin in dried tracheal exudate received on Dec. 5, 1938, from Dr. C. A. Brandly, then at the University of Illinois. On December 7, the material was suspended, filtered through a Berkefeld V candle, and inoculated into eggs. Unlike the previous strain, this one has killed embryos rather consistently from the first passage in eggs and has shown other minor differences which may be of no consequence. The strain was carried through only 12 passages and then stored in a dried state in 1940. Identity with bronchitis virus by neutralization or cross-immunity tests has never been made in this laboratory.

In a dried state as shown in table 5, this virus has retained its activity after being held for 3,021 days, that is, for a considerably longer period than the B strain of bronchitis.

In a frozen state, a sixth passage harvested June 12, 1939, was active sixty-five days later, Aug. 16, 1939.

The Williamson strain (WS) was recovered from chicks affected with a respiratory disease. Tracheal exudate was filtered through a Berkefeld V filter and inoculated into eggs on Feb. 9, 1938. The changes produced in embryos resemble, in general, those seen in embryos dead of bronchitis virus infection, but as in the case of the previous strain, identity has not been established by acceptable methods. The virus had undergone 23 passages by 1940 when it was dried for storage.

In a dried state, as shown in table 5, this virus has retained its activity for at least 3,077 days. In a frozen state, nineteenth passage material harvested Aug. 21, 1939, was active when tested Dec. 1, 1939, or 102 days later.

SUMMARY

1) Various passages from the twenty-first to the 157th of one strain of laryngotracheitis virus were found to be active, as determined by chorioallantoic inoculation into embryonating chicken eggs, after being held in a dried state from 311 to 3,359 days. A second strain which produced progressively smaller gross lesions on serial passage was active after being held in a dried state 927 days but not at 1,052 days.

2) A strain of fowlpox passaged in chicken eggs was found to be active after being held in a dried state up to 3,598 days. The same strain adapted to duck eggs was active in one series after being held in a dried state up to 1,928 days, and in a second series, up to 1,687 days.

3) Pigeon pox virus passaged in chicken eggs and held in a dried state was active up to 3,605 days except one sample which had been held 2,602 days. The same strain adapted to duck eggs was found to be active when held in a dried state up to 1,099 days. However, a second strain of pigeon pox propagated in chicken eggs was found to be inactive when a tenth passage was tested after being held 2,574 days in a dried state.

4) Dried samples representing the twenty-first and fifty-fourth passages of one strain of canary pox virus were found to be active after being held 2,571 and 1,106 days, respectively. A dried sample of the twelfth passage of a second strain was active after being held 2,571 days.

5) Dried turkey pox virus was found to be inffective after being held up to 1,468 days.

6) Material from various passages of bronchitis virus was tested after being held in a dried state from 232 to 3,053 days, but infectivity could not be demonstrated in material held more than 684 days.

7) Of two bronchitis-like strains, one (Brandly) retained its activity after being held in a dried state for 3,021 days and the other (Williamson) after being held 3,077 days.

References

[1]Beaudette, F. R., and Hudson, C. B.: The Viability and Immunizing Value of Egg-Propagated Laryngotracheitis Virus. J. A. V. M. A., *95*, (1939): 333-339.

[2]Beaudette, F. R., and Hudson, C. B.: Cultivation of Pigeon-Pox Virus on the Chorioallantoic Membrane. J. A. V. M. A., *93*, (1938): 146-150.

[3]Beaudette, F. R., and Hudson, C. B.: Egg Propagation of Turkey Pox Virus. Poult. Sci., *20*, (1941): 79-82.

The Effect of Certain Chemical Agents on the Virus of Newcastle Disease of Chickens

CHAS. H. CUNNINGHAM, B.S., M.S., D.V.M.
East Lansing, Michigan

STUDIES of the effect of certain chemical agents on the virus of Newcastle disease [pneumoencephalitis] of chickens were undertaken to obtain information which might apply to disinfection in practice. Similar studies have been recently reported by Tilley and Anderson.[1]

PROCEDURE

A strain of the virus (Acc. 46-967) which had been isolated from the filtrate of an emulsion of lung and tracheal material from a natural outbreak of Newcastle disease in chickens was used as inoculum. This virus had been originally established and cultivated in the allantoic sac of embryonated chicken eggs through 15 passages and was capable of killing all embryos by the end of the second day following inoculation. The virus suspension employed in the tests consisted of pooled allantoic fluid from embryos dead on the second day following inoculation *via* the allantoic sac route. Aliquot samples of the pooled virus suspension were stored at –35 C. and taken out as needed. The procedures employed in these studies were similar to those previously reported by Cunningham and Stuart.[2] The tests were conducted with 10- to 12-day embryos but, in the tests, all embryos employed for a particular chemical agent were of the same age. The site of injection in the allantoic chamber was determined by transillumination of the egg and selection of an area of the chorioallantoic membrane free of large blood vessels, about 3 mm. below the base of the air cell. A small hole was drilled through the shell, without piercing the shell membrane, by means of a drill attached to the chuck of an electric motor. Tincture of metaphen was painted over the hole in the shell and allowed to dry before the eggs were inoculated. After injecting the test solution into the allantoic chamber of each egg of the series, using a B-D Yale, 1-cc. capacity, tuberculin syringe fitted with a 27-gauge, ½ in. needle, the holes in the shells were sealed with melted paraffin and the eggs returned to the incubator. All incubation was at 99 F. in an electric, forced-draft incubator.

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The test solutions were at room temperature when mixed. In all tests, 1 part of the virus suspension was added to 9 parts of the test solution, thoroughly mixed, and allowed to stand for three minutes before injection in 0.05-cc. amounts into the eggs. In some instances, the mixture was injected into the eggs directly or after dilution with sterile distilled water to a chemical concentration which previous experiments had shown to be nontoxic for the embryo. In no instance was the virus diluted to such an extent as to influence its lethal action on the embryo.

After inoculation, the eggs were candled daily. Survival of the embryo for six days was considered as evidence that the material injected did not contain active virus.

The concentration of the virus suspension employed was determined by injecting 0.05-cc of tenfold serial dilutions into eggs and selecting as the titer the highest dilution of the virus which killed 50 per cent or more of the embryos inoculated. The titer was the 10^{-8} dilution and there were considered to be 10^{8} embryo m.l.d.'s in 0.05 cc. of the original suspension.

RESULTS

The results of the tests are recorded in table 1. The agents tested were prepared as percentage solutions with a few exceptions, which were prepared as dilutions. The following agents completely inactivated the virus during the three-minute reaction period: ethyl alcohol, 95 per cent and 70 per cent; tincture of metaphen (1:200), undiluted; mercuric chloride, 1:1,000; tincture of zephiran (1:1,000), undiluted; phemerol (3%), undiluted; disilyn (10%), 1:512; phenol, 3 per cent; liquor cresolis saponatus, 3 per cent; lysol, 3 per cent and 1 per cent; creolin Pearson, 3 per cent and 1 per cent; clorox, 20 per cent and 5 per cent; tincture of iodine, 2.5 per cent and 1 per cent; and sodium hydroxide, 5 per cent and 2 per cent.

The following agents did not completely inactivate the virus during the three-minute reaction period, but they were considered as being effective against the virus: roccal (10%), 1 per cent; phemerol (3%), 33 per cent; liquor cresolis saponatus, 1 per cent; potas-

sium permanganate, 1:1,000; and formalin, 10 per cent.

The following agents were without effect on the virus during the three-minute reaction period: ethyl alcohol, 40 per cent and 25 per cent; tincture of metaphen (1:200), 1 per cent; merthiolate (1:1,000), undiluted and 10 per cent; mercuric chloride, 1:10,000; tincture of zephiran (1:1,000), 1 per cent; roccal (10%), 0.1 per cent; phenol, 1 per cent; clorox, 1 per cent; potassium permanganate, 1:10,000; tincture of iodine, 0.1 per cent and 0.01 per cent; formalin, 1 per cent and 0.01 per cent; sodium hydroxide, 0.1 per cent; and boric acid, 4 per cent.

DISCUSSION

In this study and the report of Tilley and Anderson,[1] the criterion of the effectiveness of the several agents against the virus was the survival of the embryos during a definite observation period following inoculation with a mixture of the virus and agent, but the procedures varied in several respects, *viz.*, concentration of the agent, proportions of the virus and agent in the mixture, reaction pe-

TABLE I—Results of Inoculations of Eggs with Mixtures of Newcastle Disease Virus and Certain Chemical Agents (Reaction Period Three Minutes. Inoculum 0.05 cc.)

	Concentration	Subsequent dilution of mixture	Mortality in 6 days
Ethyl alcohol	95%		0/10
Ethyl alcohol	70%		0/10
Ethyl alcohol	40%		8/8
Ethyl alcohol	25%		10/10
Tincture of metaphen (1:200)	und.	1,000	0/10
Tincture of metaphen (1:200)	1%	10	10/10
Merthiolate (1:1,000)	und.		10/10
Merthiolate (1:1,000)	10%		10/10
Mercuric chloride	1:1,000	100	0/10
Mercuric chloride	1:10,000	10	10/10
Tincture of zephiran (1:1,000)	und.	100	0/10
Tincture of zephiran (1:1,000)	1%		10/10
Roccal (10%)	1%		1/10
Roccal (10%)	0.1%		10/10
Phemerol (3%)	und.	50	0/10
Phemerol (3%)	33%	10	1/10
Disilyn (10%)	1:512		0/10
Phenol	3%	100	0/10
Phenol	1%	100	10/10
Liquor cresolis saponatus, U.S.P. XII	3%	100	0/10
Liquor cresolis saponatus, U.S.P. XII	1%	100	1/10
Lysol	3%	100	0/10
Lysol	1%	100	0/10
Creolin Pearson	3%	100	0/10
Creolin Pearson	1%	100	0/10
Clorox	20%	10	0/10
Clorox	5%		0/10
Clorox	1%		10/10
Potassium permanganate	1:1,000	10	1/9
Potassium permanganate	1:10,000		9/9
Tincture of iodine, U.S.P. XII	2.5%	2,500	0/10
Tincture of iodine, U.S.P. XII	1%	1,000	0/10
Tincture of iodine, U.S.P. XII	0.1%	100	10/10
Tincture of iodine, U.S.P. XII	0.01%		7/7
Formalin	10%	5,000	2/10
Formalin	1%	500	10/10
Formalin	0.01%	10	10/10
Sodium hydroxide	5%	500	0/10
Sodium hydroxide	2%	500	0/10
Sodium hydroxide	0.1%		9/9
Boric acid	4%		10/10

riod, subsequent dilution of the mixture prior to inoculation of the embryos, amount of inoculum, numbers of embryos per tests, and the length of the observation period.

Selection of comparative tests of the same concentration of chemical agents from the data of Tilley and Anderson[1] would indicate that sodium hydroxide, 2 per cent, and liquor cresolis saponatus, 1 per cent, were effective against the virus during the five-minute reaction period. The data in the present study would indicate that sodium hydroxide, 2 per cent, and liquor cresolis saponatus, 1 per cent, were effective against the virus during a three-minute reaction period. Phenol, 3 per cent and 4 per cent, was effective against the virus during the three- and five-minute reaction periods, respectively, of each study; but phenol, 1 per cent and 2 per cent, was without effect.

Summary

A number of chemical agents were tested for their effect on an egg-propagated strain of the virus of Newcastle disease of chickens [pneumoencephalitis] during a reaction period of three minutes. Following the reaction period, the virus-chemical mixture was injected into the allantoic chamber of embryonated chicken eggs. Some of the mixtures were injected into the eggs directly, while others were diluted with sterile distilled water to a chemical concentration which previous experiments had shown to be nontoxic for the embryo. Survival of the inoculated embryos for six days was considered as evidence that the material injected did not contain active virus. The results of both effective and ineffective agents are presented.

References

[1]Tilley, F.W., and Anderson, W.A.: Germicidal Action of Certain Chemicals on the Virus of Newcastle Disease (Avian Pneumoencephalitis). Vet. Med., *42*, (1947): 229-230.

[2]Cunningham, C.H., and Stuart, H.O.: The Effect of Certain Chemical Agents on the Virus of Infectious Bronchitis of Chickens. Am. J. Vet. Res., *7*, (1946): 466-469.

Spontaneous Lymphocytoma in a Flock of Chickens

CARL OLSON, JR., D.V.M., M.S., Ph.D.
Lincoln, Nebraska

VARIOUS REPORTS[1] have been given on the incidence of neoplastic diseases in chickens. The most common tumor is lymphocytoma, as defined by Feldman and Olson.[1] The incidence of this neoplasm will vary from one flock to another, depending upon various factors. Some of these factors are probably genetic,[2,3] and environment has been suggested as having an influence.[4,5] Probably other unknown factors are also involved. The common association of lymphocytoma with so-called "leucosis complex"[6] leads to confusion of the problems of incidence of lymphocytoma.

In the summer of 1941, attention was drawn to a flock of chickens in which there seemed to be unusual loss from lymphocytoma. This flock was made the subject for investigation on the occurrence of the disease and flock mortality. An attempt was also made to control the apparent epizoötic by experimental immunization with a transmissible lymphoid tumor. These observations constitute the basis for this report.

HISTORY OF THE FLOCK

On April 30, 1941, a group of Rhode Island Red pullets, 10 weeks old, were purchased from a breeder of poultry. These were placed on a range where chickens had not been for three years. The range area was sandy ground and well drained. The water supply was from a brook. There were no other poultry yards on it for a distance of at least a mile up stream. No unusual mortality had occurred until the birds were about 6 months old, or about ten days prior to the owner's visit to the laboratory. Then a few were found dead or dying. These losses were at a rather slow rate.

On August 26, the owner brought 4 birds to the diagnostic laboratory for examination. All of these birds had lymphocytoma. The farm was visited the next day and arrangements made to study the flock. The study includes the 4 birds. They were placed on two floors of a laying house; the quarters were thoroughly cleaned and the walls, floors, and ceilings whitewashed. Fresh, clean shavings were spread on the floor. The dropping pits under the roosts were protected by a screen. The usual feeding and management practices for good laying flocks were followed.

MATERIALS AND METHODS

The flock was visited at periodic intervals, and all dead and sick birds were examined for disease. Any lesion suspected of being neoplastic was examined by histologic section to establish the character of the process. The cause of death was established, so far as possible, to provide data on mortality. Observations were discontinued March 8, 1942.

On September 2, approximately half the chickens of the flock under observation received 0.5 cc. of frozen tumor preparation in the breast muscle. The birds were legbanded for identification. On one floor of the laying house, 123 chickens received the tumor preparation, and 125 birds were untreated controls. On the other floor, 101 chickens were injected, and 116 remained as controls.

Tumor material was prepared and tested as follows: 12-day growth of tumors in the breast muscles of 5 chickens was pooled and minced; 130 cc. of tissue was obtained and mixed with infusorial earth suspension 1:1,000 and 130 cc. modified Ringer's solution. This freshly prepared material caused tumors in all of the 5 birds in which it was injected

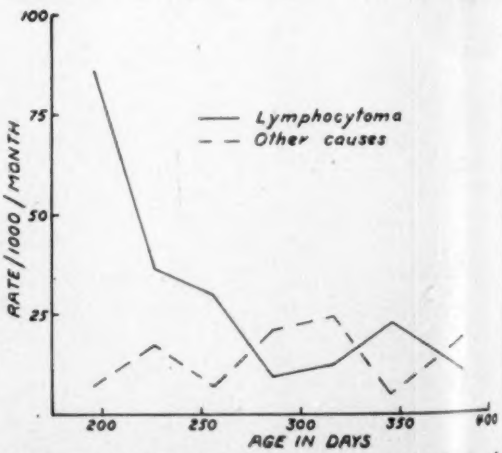


Chart 1—Mortality from lymphocytoma in a flock of 478 chickens compared to mortality from all other causes.

Contribution No. 628 of the Massachusetts Agricultural Experiment Station, Amherst, where the work was done.

Dr. Olson is in the Department of Animal Pathology and Hygiene, University of Nebraska, Lincoln.

(0.5 cc. dose). Previous work[7] has shown that if the lymphoid tumor were properly attenuated by freezing, the growth activity could be markedly reduced and an immunizing quality retained. The preparation for use in the flock was frozen at –4 C. and thawed daily for five days. It was thawed for the last time and injected on September 2. Five laboratory chickens were inoculated with this preparation, and no growths resulted. Twenty-one days later their immunity was challenged with active tumor preparation, and tumors occurred in all cases.

RESULTS

During the 196-day observation period, 107 chickens died or were in a moribund state at the time of examination. Lymphocytoma was found in 65. The 42 birds dying of other causes suffered from miscellaneous illnesses (14 from infection, 9 from cannibalism, 6 from other forms of neoplasia, 1 from fowl paralysis, and 12 from a variety of other causes). Other forms of neoplasia observed were: 2 instances of embryonal nephroma, 1 of leucosis, 1 of myelocytoma, 1 of neurogenic sarcoma, and 1 of hemangioma. In addition to lymphocytoma, 1 bird had embryonal nephroma, 1 a leiomyoma, and 1 had a hypernephroma. The chicken dead from myelocytoma also had an embryonal nephroma.

During the ten-day interval prior to vaccination with frozen tumor pulp, the incidence of lymphocytoma was extremely high. The mortality was calculated at 85.4 birds per 1,000 per month. During the next two months, the mortality from lymphocytoma was less, though still considerably over the mortality from all other causes. During the last four months of the study, the mortality varied from 9.4 to 22.7 birds per 1,000 per month. This was approximately the same as mortality from all other causes. Lymphocytoma occurred at about the same rate in the chickens on both floors of the laying house.

The character of lymphocytoma observed is indicated in table 1. There were 37 cases of the diffuse type of lymphocytoma, 22 with nodular lymphocytoma, and 6 represented a combination of the diffuse and nodular form. Among the 224 chickens that had been injected with frozen tumor preparation, there were 27 cases of lymphocytoma. These were 16 of the diffuse type, 10 nodular, and 1 of the combined diffuse and nodular form. Among the 241 uninoculated control chickens, there were 26 cases of lymphocytoma. These consisted of 15 diffuse, 8 nodular, and 3 of the combined diffuse and nodular type. The tumors occurred in both inoculated and control groups at approximately the same rate. That is, the greatest incidence was in the first two months of the observation period. None of the birds receiving frozen tumor material showed any reaction at the site of inoculation. Several months after the actual observations were discontinued, the flock owner reported that there had been no further unusual mortality. The following year, stock of a similar type was purchased from the same breeder and handled in approximately the same manner without unusual mortality.

DISCUSSION

The high incidence of lymphocytoma in this flock at the beginning of the observation period would suggest an epizoötic. Unfortunately, exact data on mortality were not available at the beginning of the episode. The decline in incidence was quite precipitous over a two-month period (chart 1). The losses then continued at a relatively low rate, although some variations were observed. A striking feature was the relative absence of other tumors or conditions sometimes associated with lymphocytoma. Conspicuous by their low incidence were fowl paralysis, myelocytoma, and transmissible leucosis. Probably there is no hypothetical "normal" rate of incidence for lymphocytoma in chickens. Olson and Bullis[8] found 6.41 per cent of 2,304 chickens affected with lymphocytoma. These birds were over 6 weeks old and had been submitted for the routine diagnosis of suspected disease. Campbell,[9,10] in a comparable study in England, found 113 of 1,692 chickens examined over a five-year period affected with lymphocytoma, an incidence of 7.8 per cent. In the flock studied, 107 birds were examined for disease, and 60.7 per cent had lymphocytoma, nearly ten times the number found in the routine of the diagnostic laboratory. The average age of chickens with lymphocytoma in the Olson and Bullis series was 34 weeks or slightly more than that of the flock at the beginning of the study.

Olson and Bullis[8] suggested that the different forms of lymphocytoma might be the

result of an interplay between the invasiveness of the tumor and the resistance of the host against neoplasia. One might, therefore, anticipate a relatively larger number of the more malignant, diffuse lymphocytoma during the earlier part of the epidemic and, conversely, a larger number of nodular lymphocytoma in the latter part of the epidemic. This was not observed as may be seen from table 1. This theory, however, would be sound if one had a single massive exposure to a hypothetic tumor-producing agent which was responsible for the apparent epidemic. Preparations of fresh tumor material from 5 cases of lymphocytoma originating in the flock were tested for transplantability and immunizing ability. These trials were made from August 28 to October 4, when the mortality from lymphocytoma was still high. The recipients of tumor material were unrelated to the flock studied and were from 31 to 47 days old. In 4 cases, neoplasia from the liver were used and in the fifth case a subcutaneous lymphocytoma. None of the implants grew, and there was no significant immunity produced against a transmissible lymphoid tumor. Details of these experiments have been previously reported.[11]

TABLE 1—Mortality from Lymphocytoma in a Flock of Chickens

Age (days)	Interval of observation (months)	No. in flock	Mortality					
			Lymphocytoma					
			diffuse	diffuse and nodular	nodular	total	Other causes	Total
187-196	.33	478	6	2	4	12	1	13
197-226	1	465	10	1	6	17	8	25
227-256	1	440	7	1	5	13	3	16
257-286	1	424	2	0	2	4	9	13
287-316	1	411	2	2	1	5	10	15
317-346	1	396	7	0	2	9	2	11
347-383	1.23	385	3	0	2	5	9	14
Total	6.56	478	37	6	22	65	42	107

The failure of the frozen transmissible tumor preparation to influence the incidence of the disease in the flock probably should have been expected, since the preparation used was incapable of inducing immunity against the same tumor in 5 experimental chickens in the laboratory. Its immunizing ability probably had been destroyed by the rigorous freezing and thawing. This test suggests that a demonstrable immunizing power must be present if any good results are to be obtained from inoculation with a tissue preparation.

The combination of neoplastic diseases found in several birds is not unusual[1] and probably has no specific significance in connection with the study.

SUMMARY

A flock of chickens was observed for six and one-half months, during which there was a subsidence of what appeared to be an epizoötic of lymphocytoma. The lack of other neoplastic diseases and fowl paralysis sometimes associated with lymphocytoma was noteworthy. Five lymphocytomas from this flock were not transplantable to experimental chickens. A preparation of a transmissible lymphoid tumor inactivated by alternate freezing and thawing was ineffective in controlling the incidence of spontaneous lymphocytoma.

References

[1]Feldman, W. H., and Olson, C.: Neoplastic Diseases of the Chicken. Diseases of Poultry, by Biester and Devries. Iowa State College Press, Ames, (1943): 523-597.

[2]Waters, N. F., and Prickett, C. O.: The Development of Families of Chickens Free of Lymphomatosis. Poult. Sci., *23*, (1944): 321-333.

[3]Hutt, F. B., Cole, R. K., and Bruckner, J. H.: A Test of Fowls Bred for Resistance to Lymphomatosis. Poult. Sci., *24*, (1945): 564-571.

[4]Barber, C. W.: The Effects of Environment on the Incidence of Avian-Leukosis Complex Lesions Among Resistant and Non-resistant Chickens. Cornell Vet., *33*, (1943): 78-84.

[5]Hutt, F. B., Cole, R.K., Ball, M., and Bruckner, J. H.: A Relation Between Environment to Two Weeks of Age and Mortality from Lymphomatosis in Adult Fowls. Poult. Sci., *23*, (1944): 396-404.

[6]Jungherr, E.: Tentative Pathologic Nomenclature for the Disease and/or the Disease Complex Variously Designated as Fowl Leucemia, Fowl Leucosis, Range Paralysis, Iritis, Lymphomatosis, Lymphocytoma, Neurolymphomatosis, Leucotic Tumors, Leucemoid Diseases. Am. J. Vet. Res., *2*, (1941): 116.

[7]Olson, C.: Immunization Against a Lymphoid Tumor of the Chicken. I. Attenuation by Freezing. Cornell Vet., *35*, (1945): 221-230.

[8]Olson, C., and Bullis, K. L.: A Survey and Study of Spontaneous Neoplastic Diseases in Chickens. Massachusetts Agric. Exper. Sta., Bull. 391, (1942): 56.

[9]Campbell, J. G.: Neoplastic Disease of the Fowl with Special Reference to Its History, Incidence, and Seasonal Variation. J. Comp. Path. and Therap., *55*, (1945): 308-321.

[10]Campbell, J. G.: Personal Communication.

[11]Olson, C.: Immunization Against a Lymphoid Tumor of the Chicken. IV. Use of Miscellaneous Tissues. Cornell Vet., *39*, (1947): 231-240.

Naturally Acquired Passive Immunity to Infectious Bronchitis in Chicks

E. L. JUNGHERR, VET. DIPL., D.M.V., and NAOMI L. TERRELL, B.A.
Storrs, Connecticut

IN BIRDS, the egg yolk appears to be the principal medium for the transference of passive immunity from the parent stock to the offspring. This important fact, together with a review of the pertinent literature, was reported for Newcastle disease [pneumoencephalitis] in chickens by Brandly, Moses, and Jungherr.[2]

That such passive immunity in chicks may be responsible, in part, for some of the unexplained factors in the occurrence and spread of Newcastle disease, has been pointed out by Jungherr and Terrell.[3]

The present work is concerned with similar observations on congenital immunity to infectious bronchitis in chickens. Thus, the scientific foundations are given for protecting brooder chicks up to the age of about 4 weeks, by active immunization of the parent stock, against the ravages of the two most important respiratory diseases of poultry.

MATERIALS AND METHODS

The infectious bronchitis virus used in this study was originally isolated and egg-adapted by Dr. F. R. Beaudette. It was received in the form of allantoamnionic fluid through the courtesy of Dr. Charles H. Cunningham and subpassaged in 10-day embryonating chicken eggs by allantoic chamber inoculation. The extra-embryonic fluid was harvested from embryos succumbing to a 1:5 dilution of the virus within forty-eight hours postinoculation. Seed virus was held at –20 C. for a period not exceeding three weeks.

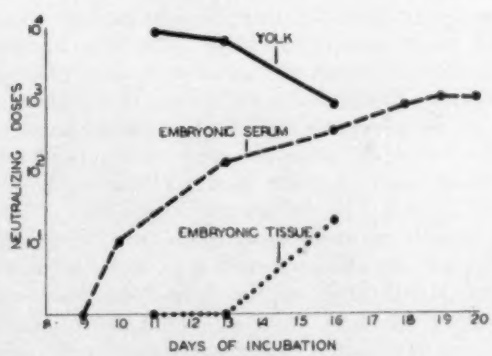
Of the materials tested for antibodies, serum was used undiluted; yolk pools from 4 eggs were diluted 1:5; egg content minus albumen and yolk was used undiluted after mechanical blending and centrifugation. All dilutions were made in sterile 1 per cent tryptose broth pH 7.

Neutralization tests were conducted in 10-day embryonating chicken eggs with bacteriologically sterile ingredients. Virus dilutions and materials to be tested for antibodies were mixed in equal quantities and injected immediately into the allantoic chamber of 3 eggs per dilution, the amount of the inoculum being 0.1 cc. per egg. The eggs were candled twice daily. The end point of the test was based upon a 50 per cent mortality of embryos in the respective egg groups. For controls, the virus was used undiluted, and diluted 1:5, and 10^{-1} through 10^{-5}, and in combination with known positive and negative serums. For actual neutralization tests, the virus was used undiluted, and diluted 1:5, 10^{-1} and 10^{-2}.

A routine neutralization test for infectious bronchitis in eggs using undiluted virus was first discussed by Van Roekel.[5] The corresponding test here was similar to that for Newcastle disease as outlined by Minard and Jungherr,[4] and standardized in a recent directive of the Bureau of Animal Industry.[1] However, the quantitative neutralization test on materials containing infectious bronchitis antibodies gave less consistent results than the corresponding test for Newcastle disease. These difficulties appeared to be due to the initial low titer of the virus and also to the variability of the titer. Although dispersability and interference phenomena of, and

CHART 1

AVERAGE NEUTRALIZING DOSES IN EMBRYONATING EGGS FROM IMMUNE FLOCK



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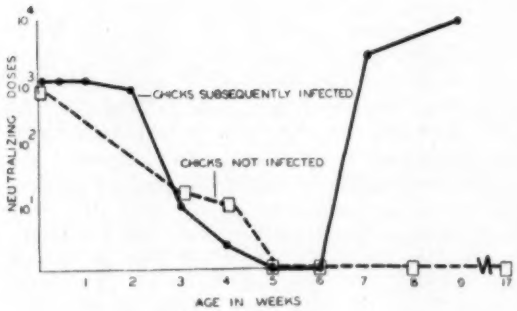
From the Department of Animal Diseases, Storrs Agricultural Experiment Station, University of Connecticut, Storrs.

unsuitable storage conditions for, the virus were suspected as being responsible for the titer variations, a definite explanation is lacking at the present time. On the other hand, all-or-none tests for the presence or absence of neutralizing antibodies were on the whole clear cut; furthermore, consecutive tests at various intervals exhibited differences in magnitude which followed an unmistakeable trend.

CHART 2

AVERAGE NEUTRALIZING DOSES IN CHICKS FROM IMMUNE FLOCKS



RESULTS

The results of the neutralization tests on incubating eggs from an immune flock are presented in table 1 and chart 1.

The parent stock was located in the University poultry plant and consisted of about 1,000 10-month-old Barred Rocks which had been immunized to infectious bronchitis by successful artificial exposure to a known bronchitis virus.

Six serum pools from 4 chicks each, obtained prior to the virus exposure, failed to show neutralizing antibodies. Five serum pools from 4 birds each, obtained one month postexposure, showed 10^3 or $>$ neutralizing doses (n.d.'s) for infectious bronchitis. Furthermore, numerous birds from this immunized stock received from the poultry plant at various times over a period of fifteen months, consistently gave positive neutralization tests. Twelve yolk pools from 4 dozen eggs collected four months postvaccination showed 10^3 n.d.'s for infectious bronchitis. Since parallel neutralization tests for Newcastle disease on chickens and eggs were negative, the significance of the bronchitis tests was strengthened.

In studying incubating eggs, yolk, egg content minus yolk and albumen, and embryonic serums were tested separately.

Twelve yolk pools collected after eleven days of incubation showed an average of 9,583 n.d.'s while another 12 pools after thirteen days of incubation showed 8,000 n.d.'s. Eleven samples tested after sixteen days of incubation gave an average figure of 990 n.d.'s.

The three corresponding test groups on egg content minus yolk and albumen, failed to show neutralizing doses after eleven and thirteen days of incubation, whereas after sixteen days of incubation neutralizing antibodies were recognizeable at low levels, averaging 26 n.d.'s.

Two to five pooled serum samples of embryos, collected at 9, 10, 13, 16, 18, 19, and 20 days of incubation showed average neutralizing doses of 0, 10, 203, 540, 875, 1,000, and 1,000 n.d.'s, respectively. The individual pools of embryonic serum samples were obtained from 30 to 10 living embryos, depending upon the age of the embryos.

As seen in chart 1, the various egg components indicated different trends in the level of neutralizing doses with advancing stages of incubation. In the yolk, the antibodies decreased markedly toward the third trimester of the incubation period, while the reverse trend was observed in the embryonic tissues and the serums.

The results of the neutralization tests on chicks from immune flocks are presented in table 2 and chart 2.

The chicks which became subsequently infected with known infectious bronchitis virus were obtained as day-old chicks from the same parent stock which furnished the incubating eggs for the above experiment. The chicks were maintained in batteries situated in isolated quarters. An average of four pooled and, later on, individual blood samples were obtained by cardiac puncture at semiweekly intervals for the first week and at weekly intervals thereafter for a total of nine weeks.

For the entire first week, the average antibody level was 1,000 n.d.'s. At the end of the second week, it had dropped to 875 n.d.'s, by the third and fourth week to 10 and 3 n.d.'s, respectively, and for the fifth and sixth week it remained at zero.

The subsequent exposure to known infectious virus occurred at the end of the fifth

week with tracheal râles being noticeable during the entire sixth week.

By the end of the seventh week, there was a sharp reappearance of antibodies reaching an average titer of 3,700 n.d.'s and of 6,200 n.d.'s by the end of the ninth week.

The chicks which were not subsequently infected represented a part of a closed flock maintained for commercial nutritional studies. The parent stock was known to have experienced an inapparent outbreak of Newcastle disease and of infectious bronchitis according to repeated positive neutralization tests for both diseases.

TABLE 1—Neutralizing Doses for Infectious Bronchitis Virus in Incubating Eggs from an Artificially Immunized Flock

	Days of incubation	No. of samples	Neutralizing doses Min.	Max.	Ave.
Yolk	11	12	5000	10000	9583
	13	12	1000	10000	8000
	16	11	0	10000	990
Egg content minus yolk and albumen	11	12	0	0	0
	13	12	0	0	0
	16	12	1	100	26
Embryonic serum	9	2	0	0	0
	10	2	10	10	10
	13	3	10	500	203
	16	5	100	1000	540
	18	4	500	1000	875
	19	4	1000	1000	1000
	20	4	1000	1000	1000

Eleven yolk pools of 4 fresh eggs each showed an average of 10^3 n.d.'s. Nine pooled blood samples of 1-day-old chicks averaged 700 n.d.'s; random samples from individual chicks taken three and four weeks later exhibited a drop to 160 and 155 n. d.'s, respectively. Twenty-five random samples collected between the fifth and the seventeenth week failed to present demonstrable antibodies. Thus, no evidence was obtained for a spread of infectious bronchitis in this flock during the period of observation.

In comparing the subsequently infected and noninfected chicks, both showed a rapid decline in antibody level from a high at 1 day of age to a vanishing point by the fifth week. Lack of neutralizing antibodies continued in the noninfected flock for seventeen weeks, *i.e.*, the longest period of observation. In the subsequently infected chicks, however, the antibody level became reëstablished within two weeks following exposure to known bronchitis virus and maintained itself at a higher level than was noted at hatching time. The early decline in antibody level was consistent with that to be expected in congenital passive immunity, while the reëstablishment of a high antibody level in the subsequently infected chicks denoted an expression of active immunity.

Field Observations

In support of the foregoing experimental results, a limited number of field observations on congenital immunity to infectious bronchitis in eggs and chicks may be of interest.

In a flock of 30,000 birds, a lot of 4,000, 7-month-old Barred Rocks and an equal number of Rhode Island Reds were known to have suffered an active outbreak of Newcastle disease during the brooder stage and to have been subjected to artificial inoculation for

TABLE 2—Neutralizing Doses for Infectious Bronchitis Virus in Chicks from Immune Flocks

Age (weeks)	Chicks subsequently infected				Chicks not infected			
	No. of samples	Min.	Max.	Ave.	No. of samples	Min.	Max.	Ave.
0.14	4	1000	1000	1000	9	500	1000	700
0.5	4	1000	1000	1000				
1	4	1000	1000	1000				
2	4	500	1000	875				
3	4	10	10	10	3	0	500	160
4	4	1	10	3	4	10	500	155
5	4	0	0	0	11	0	0	0
6	4	0	0	0	1	0	0	0
7	3	100	1000	3700				
8					7	0	0	0
9	4	5000	10000	6200				
17					6	0	0	0

bronchitis at the age of 4 months. Eight yolk pools of 4 fresh eggs, each representing four pens, were tested when the birds were 7 months old. The average titer of the yolk for bronchitis neutralizing antibodies was 10^3 n.d.'s and was considered evidence of successful inoculation.

A flock of 2,700 Rhode Island Reds of various ages became affected with a severe respiratory disease in July, 1946. Serum neutralization tests on 4-month-old birds were positive for both Newcastle disease and bronchitis, but a virus could not be isolated. Two months later, 85 pullet eggs were obtained and tested in 18 yolk pools, all of which were positive for both Newcastle disease and infectious bronchitis antibodies the titers in both instances averaging 10^3 n.d.'s.

Of a flock of 2,000 10-month-old Rhode Island Reds and 1,400 8-month-old Barred Rocks, the latter developed severe respiratory symptoms accompanied by a drop in egg production. Serum neutralization tests on the affected stock were negative for Newcastle disease and positive for bronchitis (10^3 n.d.'s). One dozen eggs collected one month after the outbreak gave similar neutralization reactions.

The above positive neutralization tests in eggs were contrasted by failure to find antibodies in three yolk pools from 1 dozen eggs, obtained from a flock of 600 8-month-old Barred Rock crosses which had come down with an acute respiratory disease two days previously.

That congenital immunity in chicks may be common under field conditions was indicated by the following cases.

A flock of 5,400 6-day-old sex-linked crosses suffered an early mortality of 370 chicks due to field encephalomalacia. Four blood samples from live, affected chicks, obtained for the purpose of ruling out Newcastle disease, were strongly positive for infectious bronchitis but negative for Newcastle antibodies. The chicks had been purchased from a commercial hatchery and, thus, no history was available regarding respiratory diseases of the parent stock.

On another poultry plant, 3,000 2-week-old sex-linked chicks sustained a mortality of about 30 in four days. The symptoms in live specimens were suggestive of the nervous form of Newcastle disease, while histologic examination indicated field encephalomalacia. Three blood samples revealed 10^3 n.d.'s for infectious bronchitis virus and none for Newcastle disease. The source of the chicks was not divulged.

A flock of 2,000 15-day-old Rhode Island Red chicks experienced a loss of approximately 1 per cent, mostly due to corneal ulcers associated with excessive lacrimation. Five serum samples tested disclosed bronchitis antibodies up to 10^3 n.d.'s while tests for Newcastle disease were negative.

These random case histories indicate that neutralizing antibodies for infectious bronchitis may be found frequently in eggs and brooder chicks in territories where infectious bronchitis is widespread. Demonstration of antibodies for infectious bronchitis in the yolk of shell eggs or in chicks up to the age of 3 weeks has the same diagnostic value as that in serum samples for adult birds, with regard to previous experience with the disease.

SUMMARY

A quantitative neutralization test similar to that for Newcastle disease was developed for the serologic diagnosis of infectious bronchitis. For routine purposes, an egg-adapted virus was used undiluted, and diluted 1:5, 1:10, and 1:100. The test was applied to both experimental and field material in over 250 instances and was found to give reliable qualitative results. Since the initial titer of the virus was relatively low and showed considerable variations, the quantitative results were more significant from the standpoint of trend in consecutive tests than from that of absolute magnitude.

In testing embryonating eggs from an artificially immunized flock during the second half of the incubation period, neutralizing antibodies were found to decline in the yolk with advancing development, and to increase concomitantly in the tissues and the serum of the embryo.

In testing chicks from artificially and naturally immunized parent stock, both lots were shown to maintain antibodies at high level during the first two weeks and to lose them by the fifth week. One lot of chicks remained free from detectable antibodies for seventeen weeks, the duration of the experiment. The other lot became exposed to infectious bronchitis at the age of 5 weeks, and showed a reoccurrence of antibody titer within two weeks.

The initial immunity for infectious bronchitis in both lots of chicks, as expressed by a high level of neutralizing antibodies, declined rapidly and thus identified itself as passive in nature. The secondary intense rise of antibody level in the exposed lot, as contrasted with the nonexposed lot, indicated active immunity-response to infection.

Field observations confirmed the experimental results to the extent that neutralizing antibodies were found frequently in the yolk of shell eggs and in the serum of asymptomatic brooder chicks in territories where infectious bronchitis was widespread.

Neutralization tests for infectious bronchitis on eggs and chicks show no overlapping with those for Newcastle disease. They have the same diagnostic significance as serologic tests on adult stock, with regard to past experience with the disease.

The occurrence of passive immunity in the offspring of actively immunized parent stock lays the scientific foundation for protecting brooder chicks against infectious bronchitis.

References

[1]Anonymous: The Diagnosis of Newcastle Disease. U.S.D.A., Agric. Res. Admin., BAI, Path. Div. (Aug. 15, 1946): 1-10 (mimeographed letter).

[2]Brandly, C.A., Moses, H.E., and Jungherr, E.L.: Transmission of Antiviral Activity *via* the Egg and the Rôle of Congenital Passive Immunity to Newcastle Disease in Chickens. Am. J. Vet. Res., 7 (1946): 333-342.

[3]Jungherr, E., and Terrell, N.: Observations on the Spread of Newcastle Disease. Proc. 50th Ann. U.S. Livestock San. Assoc. (1946): 158-171.

[4]Minard, E., and Jungherr, E.L.: Neutralization Tests with Avian Pneumoencephalitis Virus. Am. J. Vet. Res., 5, (1944): 154-157.

[5]Van Roekel, H.: Infectious Bronchitis Control. Proc. of the 15th Ann. Confer. Lab. Workers in Pullorum Dis. Control, Farmingdale, L.I., (1942): 7.

Discussion

Dr. Brandly.—I think we will agree that Dr. Jungherr and his coworkers are to be complimented for a very well designed and executed piece of experimental work. This adds more refinement to the status of various poultry diseases, in this instance bronchitis.

We are able, as a result of developments of this kind, to do a much better job of determining the presence of the disease as well as the status of a flock or of a baby chick with regard to susceptibility or possible resistance to bronchitis. Did you observe any increase in antibody titer of the embryo and comparable rise in resistance when you inoculated these embryos in the case of Newcastle?

Dr. Jungherr.—It has not been done.

Dr. Cunningham.—Dr. Jungherr's work is something that we have needed for a long time. In connection with Dr. Brandly's remarks, I think Dr. Delaplane showed in some of his work ten years ago that eggs from recovered birds were not suitable culture mediums for propagation of the virus.

Dr. Ronald Gwatkin (Ottawa, Ont.).—Mr. Chairman, I think that remark answers the question I was just going to ask, whether the immunizing property was higher in the incubated egg or in the fresh egg. Is it highest at that point and then coming down, or were your measurements only made after a certain period of incubation for the neutralizing substance in the yolk?

Dr. Jungherr.—If I understand your question, Dr. Cunningham, you ask whether the presence of the antibody interferes with the use of the egg?

Dr. Cunningham.—No, for routine propagation of the virus.

Dr. Jungherr.—So far, it has not interfered. I think the main reason for that is that the transference of the immune bodies from the yolk to the serum and the tissues of the embryo takes place at a time when you are testing; in other words, you inoculate the eggs when they are 10 days old, and within two or three days your test can be read. The eggs which we used came from immune stock and were used at an early age, so that the neutralizing antibodies in the embryos were not developing and, therefore, it didn't matter.

One can use eggs for Newcastle work from a Newcastle-immune flock on neutralization tests on those eggs. The reason is that it takes some time for the antibodies to go from the yolk into the tissues and into the cells of the embryos. One simply needs to use these eggs before the transfer takes place from the yolk to the embryo itself.

Dr. Osteen.—What is the significant titer in the case of a natural outbreak?

Dr. Jungherr.—Any titer which can be demonstrated for infectious bronchitis is considered significant at the present time. The reason is that the quantitative neutralization test for infectious bronchitis is not so well standardized at the present time and, of course, we have no definite and significant level on which to rely.

Copper Sulfate as a Treatment for Subclinical Trichomoniasis in Pigeons

DANIEL S. JAQUETTE, B.S., V.M.D.
Beltsville, Maryland

ALTHOUGH copper sulfate has been widely recommended as a treatment for trichomoniasis in pigeons, it has been tested critically only on a limited scale. Because both favorable[1] and unfavorable[2] results have been recorded from its use, the work reported herein was undertaken to determine, more critically than has been done heretofore, the efficacy of copper sulfate in the elimination of subclinical *Trichomonas gallinae* (Rivolta, 1878) infections from pigeons.

MATERIALS AND METHODS

The pigeons used were White Kings. Only those with naturally acquired, subclinical infections were used. They also appeared normal in all other respects at the time of treatment.

Basically, the determination of the presence or absence of infection with *T. gallinae* depended upon whether or not the organism could be demonstrated microscopically. Since the parasites are generally confined to the upper digestive tract[3, 4] in subclinical infections, material from this region was taken for direct microscopic examination and culturing. Another method of determining the presence of infection was based on the transmission of the parasite from parent to offspring at the time of feeding.[4] The birds to be tested were allowed to mate and raise squabs; if the squabs were raised free of the infection, the parents were also considered negative. In addition, some birds were killed, and smears secured at autopsy were examined for trichomonads.

One alone or various combinations of these four techniques were used for the detection of trichomonads in the experiments. A single negative examination of a direct smear or culturing material from the upper digestive tract was not considered conclusive evidence that a bird was uninfected. Only after a series of negative examinations, continued over an extended period, was a bird considered free of the infection.

In all tests, the copper sulfate ($CuSO_4 \cdot 5H_2O$) was administered in varying concentrations in the drinking water. The crystals were dissolved in tap water in tests 6 and 7 and in distilled water in the others. During treatment, the medicated water was kept in either glass or glazed earthenware receptacles except in test 7 where a metal receptacle was used.

The tests were divided into two series. In the first, tests 1 to 7 inclusive, the medicated water was administered to nonbreeding pigeons. Since the addition of copper sulfate to the drinking water of these birds resulted in a decreased consumption, a method of circumventing this reaction was sought. Thus, it was found that pairs which were feeding young would drink increased amounts of water in spite of the presence of the drug. Consequently, administration of the medicated solution to breeding pigeons constituted the second series, which included tests 8 to 11.

The duration of medication in the series of nonbreeding pigeons was arbitrarily decided upon beforehand. In the series of breeding pigeons, however, treatment was continued until each pair in a group had raised at least one clutch of squabs. In test 8, each pair raised four or five clutches; in test 9, each pair raised one or two clutches; in test 10, each pair raised two clutches; and in test 11, each pair raised but one clutch of squabs during medication.

Seven groups of nonbreeding and four groups of breeding pigeons were treated. Just prior to the start of treatment, the birds in each test were examined by direct smear for the presence of the parasite. Only positive birds were treated, except in test 10 where the cock of each pair was negative. One positive examination, immediately following cessation of medication, was considered ample evidence that the treatment had failed in that trial. Every bird found positive on a post-treatment examination was immediately segregated from the negative birds. If the examination following treatment was negative, additional periodic examinations were made until it was judged that the infection had been eliminated, or until the bird became positive.

This work was carried out at the United States Department of Agriculture, Agricultural Research Center, Beltsville, Md.

From the Zoölogical Division, Bureau of Animal Industry, Agricultural Research Administration, United States Department of Agriculture, Beltsville, Md.

Twenty-nine untreated, naturally infected birds were maintained as controls in a house to determine the persistence of the infection under flock conditions and to evaluate the efficiency of the direct smear as a means of determining the presence of the parasite.

OBSERVATIONS

Data pertaining to the treatment and its results are shown in table 1. In the series of tests using nonbreeding pigeons, only test 4 will be discussed in detail because the lower concentrations of the drug were less efficacious, and higher concentrations were markedly toxic. In the series of tests using breeding pigeons, only test 9 will be considered for the same reasons.

TABLE 1—Effect of $CuSO_4$ on Subclinical Trichomoniasis in Pigeons

Test (No.)	No. birds	Age (mo.)	$CuSO_4$/100 cc. of water (mg.)	Days treated	Freed of infection	
					(No.)	(%)
Nonbreeders						
1	2	10-12	10	38	0	0
2	25	1½-2	50	7	4	16.0
3	20	9-14	50	28	12	60.0
4	17	10-22	100	20	15	88.2
5	13	1-4	250	7	7	53.8
6	10	1-2	500	7	8	80.0
7	5	1	500	7	1	20.0
Breeders						
8	6	8½-11	25	182	4	66.6
9	14	7-15	35	87-97	12	85.7
10	4	15-17	40	76-89	4	100.0
11	6	7	50	110	6	100.0

Test 4 (Nonbreeders).—Each of the 15 birds freed of the infection was examined six times by direct smear during the sixty-five days following termination of the treatment. All were found negative. Immediately following the last negative examination, each of these birds was inoculated with approximately 3 cc. of a suspension of *T. gallinae* obtained by scraping the mucosa of the upper digestive tract of 2 infected squabs. Six and fourteen days later each of the birds was examined by direct smear and found to be positive on both examinations. This demonstrated that they had remained susceptible to infection with the organism. In addition, it presented circumstantial evidence that a latent infection had not existed during the period of negative examinations, since inoculation with a few organisms was followed by a patent infection.

Only 1 bird in this test showed clinical evidence of toxicity during treatment. This was manifested by a loss of weight and depression. Recovery occurred on the cessation of treatment. Postmortem pathology was studied only in the 15 birds which were freed of the infection. These were killed seventy-nine days after treatment was stopped and were examined macroscopically for pathologic changes. The most constant alterations were found in the liver. These consisted of small, grey, necrotic foci scattered throughout the organ. In 3 birds, these foci were so numerous that no normal tissue was apparent. The degree of liver damage in the remaining birds was variable but generally less extensive. The least damage was found in 2 birds in which only a few scattered lesions were observed. In addition to the liver damage, the kidneys of 2 birds appeared abnormal. In 1, this organ was dark and hemorrhagic, and in the other it was very pale. To what extent these pathologic changes may be attributable to the action of the copper sulfate cannot be accurately determined, since 14 of the birds had been used in previous treatment experiments with various drugs. The only bird which had not been treated previously was the one displaying the least amount of liver damage (only a few scattered lesions).

From an average pretreatment weight of 610 Gm., the birds lost an average of 7 Gm. each. This may partially be explained by the fact that the average daily pretreatment consumption of water was 60 cc., whereas the average daily consumption of medicated water was only 44.3 cc. per bird.

Test 9 (Breeders).—Each of 6 pairs of pigeons raised one clutch of squabs, a total of 11, free of the infection in the post-treatment period. These 11 squabs were examined 55 times by direct smear and found negative. Forty-five to sixty-six days following the end of treatment, smears were examined from each of the adult birds and found negative. The seventh pair of birds remained infected.

None of the adult birds showed evidence of toxicity, and the average weight increased 60 Gm. each during treatment. Ten of the birds were killed and,, at autopsy, 6 were found to have many small, white foci in the liver. One such liver was sent to the Patho-

logical Division of the Bureau of Animal Industry for a bacteriologic examination; the results were negative. Tissue from a similarly affected liver was sent to that division for histologic study. The report was as follows: "Histologic examination showed that the small white areas in the liver were lesions consisting of small necrotic centers surrounded by a relatively wide zone of inflammatory reaction containing giant cells, histiocytes, leucocytes, and lymphoid cells. No parasites or bacteria were found in the lesions examined." No definite conclusions can be drawn as to whether these lesions were caused by the copper sulfate.

In order to determine the daily, untreated water and medicated water consumption of a single pair of pigeons during various phases of the reproductive cycle, one pair was housed separately. The combined daily average consumption of water for the 2 birds was 184 cc. for seven days preceding the start of medication. The combined daily average consumption of medicated water for the same birds was 134 cc. for twenty-one days preceding the day on which the eggs of the first clutch hatched. During the twenty-six days that the 2 squabs were being fed by the old birds, this daily average consumption was 568 cc. The highest single day's consumption of the medicated water during the latter period was 875 cc.

Controls.—Twenty-nine infected, untreated birds were maintained under flock conditions as controls. The length of time they were kept under observation varied from 228 to 744 days. The number of times each bird was examined varied from four to 20. Altogether, 226 direct smears were prepared and examined; 218 were positive. Five birds appeared negative on one examination each, and a sixth bird, cock 1212, appeared negative on three nonconsecutive examinations. Only this last bird was found to be negative on the final examination made during the period when the birds were housed together. Subsequent to the time of the experiment, this bird was housed individually in order to determine whether it actually harbored a low-grade infection which might become patent at some later time. Periodic examinations made during the next three months were negative; presumably the bird had become free of the infection spontaneously. Nine of the control birds were killed and examined macroscopically; no lesions were found in any of the thoracic or abdominal viscera.

DISCUSSION

As shown in the table, the most effective concentration of copper sulfate tested on nonbreeding pigeons was 100 mg. of the drug per 100 cc. of drinking water, administered for twenty days. Fifteen of the 17 birds treated were freed of infection with *T. gallinae*. Although no pronounced symptoms of toxicity were evident, it seems probable that some liver damage resulted from treatment.

The most effective concentration tested on breeding pigeons, which did not produce any evidence of toxicity, was 35 mg. of the drug per 100 cc. of solution. Seven pairs of birds were treated, and 6 of them became free of the infection. Again it seems probable that some liver damage resulted from treatment.

The advantage of treating pigeons while they are feeding young is that they will drink greater quantities of medicated water than birds not feeding young. Because of this, concentrations too low to be effective in removing the trichomonads from nonbreeding pigeons may become effective when administered to breeding pigeons. In spite of the fact that the amounts of copper sulfate solution imbibed by breeding pigeons were greatly increased, the amounts actually assimilated by them were probably not increased, as the excess was disgorged into the mouths of the squabs.

If the trichomonads can be eliminated from the healthy carriers, these birds will then be able to produce squabs free of subclinical and, consequently, clinical forms of trichomoniasis. Pigeons which have been freed of the infection, however, remain susceptible, and unless they are segregated from infected birds and strict sanitary measures instituted to prevent introduction of the parasites, reinfection probably will occur. This must be kept in mind when contemplating treatment of a flock of pigeons. Since no treatment has yet been devised which will eliminate the infection from all birds, it is necesesary to identify those which were refractory to treatment so that they may be removed from the clean birds.

The observations on the 29 birds maintained as controls indicated that infection with *T. gallinae* may be very persistent in a flock of pigeons housed together. Although

an occasional bird may lose the infection, it is probable that under conditions of constant exposure, as those found in a flock of pigeons, such a bird would sooner or later become reinfected. Whether the infection is normally persistent in the individuals of a flock, or whether it results from a series of reinfections has not been finally determined. Since the treated birds in these tests were housed under conditions similar to those of the controls, it is assumed that those freed of the infection became so as a result of medication.

The high percentage of positive results obtained from examining direct smears prepared from the control birds is an indication of the efficiency of this method of examination for the parasite.

Summary

1) Two series of tests were carried out to determine the efficacy of copper sulfate in the elimination of subclinical *Trichomonas gallinae* infections from pigeons. Altogether, 122 birds were treated with various concentrations of the drug administered in the drinking water. The following conclusions are drawn:

a) The most effective concentration tested for nonbreeding pigeons was found to be 100 mg. of the drug per 100 cc. of solution.

b) The concentration most effective for breeding pigeons, without producing evidence of toxicity, was 35 mg. of the drug per 100 cc. of solution.

2) Concentrations of the drug greater than 100 mg. per 100 cc. of solution for nonbreeding pigeons, and greater than 35 mg. per 100 cc. of solution for breeding pigeons, were markedly toxic.

3) The toxic manifestations resulting from administration of copper sulfate were depression, loss of weight, and possible liver damage.

4) Twenty-nine infected, untreated control birds were kept under observation for 228 to 744 days. Of 226 direct smears examined, 218 were positive.

References

[1]Miessner, Herman, and Hansen, K.: Trichomoniasis der Tauben. Deutsche tierärztl. Wchnschr., *44*, (1936): 323.

[2]Florent, A.: La Trichomoniase du Pigeon. Ann. Med. Vet., *83*, (1938): 401.

[3]Cauthen, George: Studies on *Trichomonas columbae*, a Flagellate Parasitic in Pigeons and Doves. Am. J. Hyg., *23*, (1936): 132.

[4]Stabler, Robert M.: *Trichomonas gallinae*, Pathogenic Trichomonad of Birds. J. Parasitol., *33*, (1947): 207.

The Efficacy of Methyl Bromide as a Fumigant for the Prevention of Cecal Coccidiosis in Chickens

W. A. BONEY, JR., B.S., D.V.M.
College Station, Texas

THE PURPOSE of this paper is to report the effects of methyl bromide as a fumigant on sporulated oöcysts of *Eimeria tenella*, the causative agent of cecal coccidiosis of chickens, under natural conditions, using soil and cane pulp as a litter. Good results are often obtained in preventing an acute outbreak as well as slowing up acute infections already established, by limiting the intake of sporulated oöcysts, since the birds will develop gradual immunity to coccidia provided they do not pick up massive infections at any specific time. The intake of sporulated oöcysts may be limited either by their destruction or by removal at regular intervals. The intermittent removal is usually laborious and costly in that much good litter must be removed and destroyed. Therefore, if some good agent could be found that would inactivate the oöcysts in the litter, the infection could be prevented or held to a minimum while allowing immunity to develop normally. In this way, the expense involved would not be so great, and good litter could be used for longer periods.

The properties of methyl bromide and its action on certain free-living nematodes, parasitic nematodes of cattle and hogs, and coccidial oöcysts as described by Taylor and McBeth,[1] Livingston,[2] Swanson and Taylor,[3] Andrews, Taylor, and Swanson,[4] suggested its use as a fumigant for the destruction of sporulated oöcysts and for the prevention of coccidiosis. It was, therefore, used experimentally, simulating as nearly as possible conditions existing on farms.

MATERIALS AND METHODS*

Methyl bromide (bromethane, CH_3Br; mol. wt. 94.94., sp. gr. 1.732., m.p. –93 C., b.p. 4.5 C.) is a colorless gas at ordinary temperature and is available in the liquefied state in sealed cans. The product is colorless and practically odorless. Caution must be used in handling this product, especially in enclosures or in houses, and a gas mask must be used whenever there is danger of inhalation.

The chickens used in this experiment were 4 weeks old, having been secured from a local hatchery which had been instructed to raise them on wire floors. The floors were to be cleaned daily in order to minimize infection and thus to insure chickens as highly susceptible to coccidial infection as possible.

The sporulated oöcysts were obtained by homogenizing cecums and cecal contents of chickens previously infected with *E. tenella* in a Waring blendor. This product was mixed with equal parts of 2.5 per cent potassium dichromate solution and allowed to stand for ten days. Microscopic counts showed that each cubic centimeter of the stock suspension contained 2 million oöcysts. For use, this suspension was diluted with water so that each cubic centimeter contained approximately 100,000 sporulated oöcysts. The suspension was sprinkled on the soil and litter to provide the desired number of oöcysts indicated in each experiment.

The experiments were carried out on shady plots of soil, in range shelters, and in brooder-houses located on our experimental farm. Cane pulp and soil were used as litter on the shady plots and in the range shelters. Cane pulp litter was used in brooder-houses. The size of the plots, range shelters, and brooder-houses were 2 ft. by 3 ft., 4 ft. by 5 ft., and 10 ft. by 12 ft., respectively.

Three separate experiments were run. The methods, as described, varied little. The areas selected for the experiments were cleaned of dead plant material by the use of a garden rake. Cane pulp litter was applied to some of these plots while others were left bare. Then 2 sq. ft. of each were contaminated with *E. tenella* oöcysts. These areas were again raked in order to work the oöcysts into the top layer of the soil or into the litter. Following this, the plots, with the exception of the control plots, were fumigated with methyl bromide.

To facilitate fumigation, the contaminated areas (both soil and litter), with the exception of the brooder-houses, were covered with a sheet of Sisal Kraft paper 5 ft. long and 4 ft. wide. The edges of the paper were cov-

*Chicks and methyl bromide furnished by the Dow Chemical Company.

Poultry Veterinarian, Texas Agricultural Experiment Station, College Station.

ered with soil, and a small amount of water was sprayed over the entire cover to prevent gas leakage. The efflux tube of a methyl bromide burette (metered as a liquid) was introduced. At the end of thirteen hours, a test** was made for the presence of halogens, and positive results were obtained in all cases. The plots and houses were allowed to air for one hour after treatment was discontinued before the introduction of chickens.

After fumigation, the ground plots were enclosed with hardware cloth 24 in. high. The enclosures were then covered with Sisal Kraft paper to afford protection against the weather.

In the case where the experiment was carried out in the brooder-house, litter was contaminated with the oöcysts, and the fumigant was introduced from the outside through an efflux tube of a methyl bromide burette. The tube was introduced through a hole in the wall and the end of the tube placed in a pan on the floor. This was a space fumigation. The chickens were then placed in the house and confined to it and outside experimental areas. For the first twenty-four hours after introducing the birds, the feed was placed on the ground or litter, so that there was ample opportunity to pick up viable oöcysts. Following this, standard feeders were used.

Observations for bloody droppings were begun in each experiment on the fourth day after initial exposure and were continued for seven days. Autopsies and microscopic examinations of cecal contents for oöcysts began on the seventh day after beginning exposure.

Chickens in all experiments, except experiment 3, were subjected to autopsy on the seventh or eighth day. Survivor chickens in experiment 3 were allowed to reach the age of 11 weeks when they were sold as grade "A" fryers.

**A Frigidaire halide leak detector, using methyl alcohol was used to detect the presence of halogens.

EXPERIMENTAL

Experiment Number 1. — This being our first attempt to use methyl bromide to control coccidia, the experiment was designed so that different amounts of methyl bromide could be used on areas of uniform size to determine the effect of different concentrations on sporulated oöcysts of *E. tenella*. At the same time, controls were set up to determine whether the chickens were susceptible to the coccidia. It will be seen by examination of table 1 that seven different types of controls were set up:

1) A battery with 10 chickens uncontaminated and not fumigated.
2) A battery with 5 chickens given 40,000 sporulated oöcysts orally.
3) Plot 19 with 10 chickens on soil uncontaminated and not fumigated, to determine whether the native soil was contaminated with coccidia.
4) Plot 17 with 10 chickens on soil contaminated with 8 million sporulated oöcysts /sq. ft. and not fumigated.

TABLE 1—Effect of Methyl Bromide on Eimeria Tenella Oöcysts in Soil or Cane Litter

Confinement area	Oöcysts /sq. ft.	Meth. Br. /sq. ft. (cc.)	No. birds surviving 7 days*	Gain/bird—7 days (Gm.)	Coccidiosis on 7th day
Battery	none	none	10/10	95	Slight
19—soil	none	none	10/10	64	Moderate
17—soil	$8x10^6$	none	8/10	64	Severe
18—soil	$8x10^6$	none	8/10	36	Severe
15—soil	$8x10^6$	0.15	10/10	123	Slight
16—soil	$8x10^6$	0.4	10/10	123	None
17—soil	$8x10^6$	1.15	10/10	110	None
27—litter	none	none	10/10	86	Slight
26—litter	$16x10^6$	none	9/10	41	Severe
25—litter	$16x10^6$	0.15	10/10	86	Slight
24—litter	$16x10^6$	0.4	10/10	96	None
23—litter	$16x10^6$	1.15	10/10	96	None
Battery**	40,000	none	5/5	40	Severe

White Leghorn cockerels, 4 weeks old, confined in experimental plots and battery. Ten birds/group. Temperature 65 F., thirteen hours exposed to methyl bromide.

*Denominator is number of birds exposed and numerator is number of birds that survived.

**Control group of 5 birds each given 40,000 oöcysts orally to check infectivity of oöcysts.

5) Plot 18, same as No. 4.
6) Plot 27 with 10 chickens on litter over soil uncontaminated and not fumigated, to determine whether the cane pulp litter was precontaminated with coccidia.
7) Plot 26 with 10 chickens on litter contaminated with 16 million sporulated oöcysts/sq. ft. and not fumigated.

Soil plots 15, 16, and 17 were each contaminated with 16 million sporulated oöcysts per plot, and fumigated with 0.15, 0.4, and 1.15 cc. per square feet of total space, respectively. Plot 17 previously had been used unfumigated and gave good indication that the oöcysts were viable. This time more oöcysts were added, and it was fumigated.

Cane pulp plots 23, 24, and 25 were prepared in the same manner, except that 32 million sporulated oöcysts were used on each.

Since only the inoculated birds in the battery (control 2) became heavily infected, it is indicated that the chickens used in this experiment were susceptible to coccidial infection and that the oöcysts were viable. This is further substantiated by the observation that chickens in other control groups, which were either inoculated or picked up the infection from artificially contaminated soil or from native soil contamination, exhibited acute symptoms of coccidiosis. Results from plot 19 indicate that the native soil was contaminated, and the results on plot 27 would indicate that the litter was not previously contaminated.

Table 1 shows that the chickens on the soil and litter plots fumigated with 0.15 cc. of methyl bromide/sq. ft. showed a light infection, whereas the chickens on the soil and litter plots receiving 0.4 and 1.15 cc. of methyl bromide, respectively, showed no infection at autopsy on the seventh day, thus indicating that methyl bromide was effective in inactivating sporulated oöcysts of *E. tenella* when used in a concentration of 0.15 cc. or more/sq. ft.

On the basis of this evidence experiment 2 was designed.

Experiment Number 2.—The object of this experiment was identical with experiment 1. Three large plots, 4 ft. by 5 ft., and two small plots, 2 ft. by 3 ft., were used. The large plots facilitated the confinement and exposure of 50 chickens each instead of 10. The chickens were confined to the larger plots in small range shelters with wire floors removed. The procedure in preparation of the plots was the same as in experiment 1. Where methyl bromide was used, the amounts were either 0.15 or 0.3 cc./sq. ft. and fumigation was continued for thirteen hours. On the fourth day of this experiment, a heavy rainfall caused water to flow through all of the pens and left water standing in the pens, thus possibly providing a source of new contamination.

Three plots, 1, 2, and 5, which accommodated 50 chickens each, were prepared, and 2 sq. ft. of each were inoculated with 8 million sporulated oöcysts/sq. ft. Plot 5 was not fumigated and could be considered as another type of control since the results indicate that the sporulated oöcysts were infective and that the chickens were susceptible. In plot 5, 20 chicks were dead on the seventh day after exposure, and the remainder were heavily infected. The results of this experiment are shown in table 2. In plot 1 where 0.15 cc. of methyl bromide was used, only 3 chickens succumbed; 42 showed a light infection microscopically; and 5 showed no infection, indicating partial control of infection. In plot 2 which received 0.3 cc. of methyl bromide/sq. ft., none of the chickens showed any evidence of coccidiosis either macroscopically or microscopically. In two small plots, 6 and 7, similar to those used in experiment 1, 10 chickens were confined in each and exposed to natural or native soil infection. All chickens in plot 7, which was not fumigated, succumbed within seven days. All those in plot 6, which was fumigated with

TABLE 2—Effect of Methyl Bromide on Eimeria Tenella Oöcysts in Soil

Confinement area	Oöcysts /sq. ft.	Meth. Br. /sq. ft. (cc.)	No. birds surviving 7 days*	Gain/ bird—7 days (Gm.)	Coccidiosis on 7th day
Battery	None	None	10/10	95	Slight
5—soil	$8x10^6$	None	30/50	32	Severe
1—soil	$8x10^6$	0.15	47/50	59	Slight**
2—soil	$8x10^6$	0.3	50/50	95	None
7—soil	None	None	None	—	
6—soil	None	0.3	10/10	59	Slight**

White Leghorn cockerels, 4 weeks old, confined in experimental plots. Temperature 65 F., fumigated for thirteen hours.
*Denominator is number of birds exposed and numerator is number of birds that survived.
**Heavy rainfall on the fourth day of this experiment left water standing in and around all pens.

0.3 cc. of methyl bromide/sq. ft., survived but showed a light infection at the end of seven days.

The chickens confined to a clean, unfumigated battery brooder served as controls and only 1 showed infection, which was very slight, on the seventh day. This evidence would indicate that the minimum effective concentration of methyl bromide for the control of *E. tenella* in soils of this type lies between 0.15 and 0.3 cc./sq. ft.

Experiment Number 3.—The object of this experiment was to demonstrate the efficacy of methyl bromide as a brooder-house fumigant for the prevention of coccidiosis in chickens exposed to litter contaminated with sporulated oöcysts.

Three brooder-houses constructed of wood, including the floor, were used for the test. A bed of cane pulp litter was spread to a depth of 1½ in. in each house over the total floor area of 120 sq. ft. The litter in all houses was dampened with water to provide adequate moisture for the survival of the parasites. A suspension of sporulated oöcysts of *E. tenella* was spread over a central floor area measuring 6 ft. by 6 ft. to provide 16 million organisms/sq. ft.

cu. ft. of space. It was considered possible that sealing with masking tape did not completely close all cracks, consequently, house 1 was fumigated with 3 lb. of gas/1,000 cu. ft. to allow for slight leakage. House 3 was not fumigated and served as the control. Twenty-five chicks were placed in a clean battery, unfumigated, to serve as controls and to determine normal weight gains. The fumigation was started in the evening and allowed to run for thirteen hours. At the end of this time, the atmosphere of both houses showed a positive test for the presence of halogen. The doors and windows were open for an hour before chickens were placed in the houses.

One hundred and fifty white Leghorn cockerels, 4 weeks old, were placed in each house. During the first twenty-four hours, feed was spread on the previously contaminated litter. Standard feeders were used for the remainder of the experiment.

Precautions were taken to avoid the accidental contamination of the houses with oöcysts. No one entered the houses after fumigation. Feeding, watering, and removal of dead birds were managed from the doorway.

After seven days of exposure to infected, unfumigated litter only 39 of the 150 control

TABLE 3—Effects of Methyl Bromide Fumigation on Eimeria Tenella Oöcysts in Brooder-Houses

Confinement area	Oöcysts /sq. ft.	Meth. Br. /sq. ft. (cc.)	No. birds surviving 7 days*	Gain/bird—7 days (Gm.)	Coccidiosis on 7th day
Battery	None	None	25/ 25	104	None
3	$16x10^6$	None	39/150	–9	Severe
1	$16x10^6$	3 lbs./1000 cu. ft. 16 hrs.	149/150**	100	None
2	$16x10^6$	2 lbs./1000 cu. ft. 16 hrs.	149/150**	109	None

White Leghorn cockerels, 4 weeks old, confined in experimental plots. Temperature 65 F., fumigated for thirteen hours.

*Denominator is number of birds exposed and numerator is number of birds that survived.

**Death due to crowding.

In order to retain an effective concentration of methyl bromide, the brooder-houses were carefully sealed. This was accomplished in two ways. House 1 was sealed by covering all cracks with masking tape. (Wet newspaper can be used to seal cracks in a brooder house.) House 2 was completely covered with a gas proof tarpaulin (40 by 50 ft.), the edges of which were held down with "sand snakes" (elongated canvas sacks filled with sand).

House 2 (covered with a tarpaulin) was fumigated with 2 lb. of methyl bromide/1,000

birds survived (house 3), and these survivors showed evidence of coccidiosis. In house 1, previously sealed with masking tape and fumigated with 3 lb. of methyl bromide/100 cu. ft., 149 of the original 150 chickens remained alive. In house 2, treated with 2 lb. of methyl bromide/1,000 cu. ft. (under gas proof tarpaulin), 145 birds survived out of 150 which were started. None of the birds which died in the fumigated houses showed any evidence of coccidial infection. There were no deaths in the 25 chickens placed in the clean, unfumi-

gated battery brooder, and the chickens placed in the fumigated brooder houses showed a weight gain comparable to those in the battery brooder.

It was desirable to know whether the birds in houses 1 and 2 (fumigated) were still susceptible to coccidiosis, or if there had been a certain amount of immunity developed from the ingestion of inactivated oöcysts. To ascertain this, 5 birds were picked at random from each of houses 1 and 2, fifteen days after the beginning of this experiment, and placed in house 3 (infected and unfumigated). Five days later all 10 birds were dead and, upon autopsy, exhibited acute cecal coccidiosis.

Approximately seventeen days after the beginning of the experiment, litter was added to house 1 and stirred in with the litter already present. No litter was added to house 2, and it was not stirred. The birds were kept in these houses until they were 11 weeks old. Approximately twenty days after the beginning of this experiment, a coccidial infection gained a foothold in houses 1 and 2. It lasted for ten days and resulted in a mortality of 16 per cent in house 1 and 6.7 per cent in house 2.

SUMMARY AND CONCLUSIONS

An effort was made to determine the effects of methyl bromide used as a fumigant upon the infectivity of sporulated oöcysts of *Eimeria tenella*, the causative agent of cecal coccidiosis of chickens, under conditions closely simulating those found on the average poultry farm. For this reason, experiments were carried out using soil as the litter, cane pulp on soil, and cane pulp as litter on wooden floors of brooder-houses.

In each of three experiments, the litter or soil was contaminated with sporulated oöcysts of *E. tenella*. The rate of contamination was in some instances 8 million and in others 16 million sporulated oöcysts/sq. ft. Methyl bromide was applied to some of the contaminated areas and others were not fumigated to serve as controls. The amount of methyl bromide used (metered as a liquid) varied from 0.15 to 1.15 cc./sq. ft. of contaminated litter (soil or cane pulp).

It was found that methyl bromide was effective in inactivating sporulated oöcysts of *E. tenella* when properly applied at the rate of 0.15 to 0.3 cc./sq. ft., which is approximately 1 lb./1,000 sq. ft.

It was also demonstrated that methyl bromide would prevent infection with oöcysts from brooder-houses where cane pulp was used as a litter on a wooden floor, when 2 lb. methyl bromide/1,000 cu. ft. were properly applied.

The tests described demonstrated the possible economic value of methyl bromide as a fumigant for the control of cecal coccidiosis.

References

[1]Taylor, A. L., and McBeth, C. W.: Preliminary Tests of Methyl Bromide as a Nematocide. Proc. Helminthol. Soc. Wash., 7, (1940): 94-96.

[2]Livingstone, E. M.: Fumigation of Bulk Soil with Methylbromide for the White Fringed Beetle in New Orleans. Div. of Control in Invest., B. Entom. and Plant Quarantine, September, 1940. Mimeographed.

[3]Swanson, L. E., and Taylor, A. L.: Control of Cattle Parasitic and Free-living Nematodes by Soil Fumigation with Methylbromide. Proc. Helminthol. Soc. Wash., *10*, (1943): 1-3.

[4]Andrews, John S., Taylor, A. L., and Swanson, L. E.: Fumigation of Soil with Methylbromide as a Means of Destroying Infective Stages and Intermediate Hosts of Some Internal Parasites of Mammals. Proc. Helminthol. Soc., Wash., *10*, (1943): 4-6.

Sulfathiazole as an Aid to the Control of Fowl Cholera in Chickens and Turkeys

H. M. DeVOLT, D.V.M., M.S.
College Park, Maryland

FOWL cholera is a disease of ancient origin. The ailment long has ravaged poultry flocks without there having been developed a uniform plan of control. The discovery during recent years of a new class of drugs, collectively designated sulfonamides, has brought relief from certain ailments formerly considered difficult to control.

LITERATURE

The experimental literature contains numerous references to the effectiveness of sulfonamides against diseases of man and the domestic animals. In the field of poultry pathology, Cardoso, Nobrega, and Reis[1] reported sulfanilamide, administered individually, to be effective in reducing losses from fowl cholera. Delaplane and Stuart[2] found sulfathiazole to be efficacious in the prevention and treatment of infectious coryza. Severns, *et al.*,[3] and other investigators have shown several sulfonamides to be capable of reducing losses from pullorum in baby chicks. Hammond[4] reported sulfathiazole to be effective in combating fowl typhoid. Recently, Kiser *et al.*[5] found sulfamethazine effective in reducing losses from cholera.

Chemotherapeutic agents of this type are capable of administration in amounts sufficiently large to be effective and still preserve a reasonably wide margin of safety against toxicity. Such medicaments would seem to lend themselves readily to the control of poultry diseases, since it is more convenient to offer medicated food and drink than to establish a drug dosage suitable to the individual.

MATERIALS AND METHODS

The general plan of these laboratory experiments included the administration of sulfathiazole in feed forty-eight hours before artificial exposure to fowl cholera. The subsequent performance of experimental groups was determined by daily observation and weekly weighing. A test period of sixty days was employed.

Artificial exposure to cholera was accomplished by intravenous inoculation with virulent cultures of *Pasteurella avicida*. The minimum lethal dose (m.l.d.) of each culture was determined in preliminary trials, and an inoculating dose capable of inducing high mortality in control groups was administered. All Pasteurella strains employed in these experiments were of chicken origin and collected from Maryland or nearby states.

Sulfathiazole was administered to experimental chickens in all cases by mixing the drug with laying mash. Grain feeding was eliminated during the course of the experiments. Drug levels of 0.50 per cent, 1.0 per cent, and 2.0 per cent were studied. The total period of medication was extended to sixteen days or fourteen days after artificial exposure to the disease. Blood tests were made in each experimental group to determine the concentration of sulfathiazole in milligrams per 100 cc. of circulating blood.

The New Hampshire breed of chickens has been employed in all experiments to date. For the most part, the chickens subjected to infection have been 4 to 5 months of age, but in one experiment 1-year-old fowls were used. Equal numbers of the sexes have been included in experimental groups.

The experimental chickens were kept in wire-floored batteries or cages with 1 or 2 chickens in each compartment. A total of 366 chickens was employed in six experiments over a two-year period.

In connection with the experiments outlined, a limited number of observations on naturally infected flocks have been made.

Effect of Sulfathiazole on the Clinical Course of Fowl Cholera.—In studying the effect of sulfathiazole on the clinical course of fowl cholera, the drug was administered at three levels, namely, as 0.5 per cent, 1.0 per cent, and 2.0 per cent of the total mash feed consumed. Since the three drug levels were found to exert the same general effect on the course of experimental disease attacks, the results of all experiments with highly virulent cultures are summarized in table 1. Not in-

From the Department of Animal Pathology, University of Maryland, College Park.

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cluded in this table are 60 chickens used for one test in which the mortality was 33-1/3 per cent in controls and zero in the medicated group.

In five experiments in which a total of 271 chickens was employed, 106 controls (96.4%) became sick out of 110 as compared with 22 (13.7%) in the treated groups, totaling 161 chickens. The total number of treated chickens exceeds that of the controls for the reason that, in making comparative tests with different levels of the drug, one control group served for two treated groups. The incubation period of the experimental disease attack averaged 0.83 days in 106 untreated chickens, and 16.5 days, or 19.8 times longer, in 22 sick chickens in treated groups. The periods of clinical disease were nearly the same, 7.02 and 8.9 days, respectively, for the control and treated groups, indicating that chickens too sick to eat naturally were deprived of the benefit of treatment. There were 11 recoveries in control groups and only 2 in treated groups. The mortality was 86.3 per cent (95 chickens) in the controls and 12.4 per cent (20 chickens) in treated groups. There was thus a difference of 73.9 per cent in mortality between the two groups. The average length of life in control groups was 13.8 days or 23.0 per cent of the test period.

TABLE 1—Effect of Sulfathiazole on the Clinical Course of Experimental Fowl Cholera

			Morbidity record									
No. exptl. chickens	Resistant chickens (No.)	Resistant chickens (%)	Sick chickens (No.)	Sick chickens (%)	Ave. length incubation period (days)	Ave. length clinical period (days)	Recovery (No.)	Recovery (%)	Mortality (No.)	Mortality (%)	Ave. life (days)	Ave. life as per cent of test period
Controls, 110	4	3.6	106	96.4	0.83	7.02	11	10	95	86.3	13.8	23
Treated, 161	139	86.3	22	13.7	16.5	8.9	2	1.3	20	12.4	55.9	93.1

TABLE 2—Comparative Tests with Various Levels of Sulfathiazole Employed Against Artificially Induced Fowl Cholera

Experimental group	No. of chickens	Resistant chickens (No.)	Resistant chickens (%)	Sick chickens (No.)	Sick chickens (%)	Mortality (No.)	Mortality (%)
Control	54	4	7.4	50	92.6	46	85.1
2%	54	46	85.1	8	14.8	8	14.8
1%	30	26	86.6	4	13.3	4	13.3
0.5%	24	20	83.6	4	16.4	4	16.4

In treated groups, 66.4 per cent of the chickens received sulfathiazole at the 2 per cent level, and the other fowl were nearly equally distributed between the 0.5 per cent and 1 per cent levels.

Toxic Effects of Sulfathiazole.—Although sulfathiazole was administered at the 2 per cent level to nonlaying chickens in critical experiments without toxic effects, this level later was found too high for flocks in production. In experiments conducted in the Department of Poultry Husbandry, University of Maryland,* a flock of New Hampshire hens laying at the rate of 40 per cent became sick in three to four days after a 2 per cent mash was offered. Egg production dropped to a low point, and most of the flock did not recover for about two weeks.

Results obtained with lower drug levels would seem to indicate that the 2 per cent level is higher than ordinarily required. In preliminary tests with nonlaying chickens, this drug concentration was found to be the highest tolerated without seriously depressing feed consumption.

Comparative Tests with Various Sulfathiazole Levels.—Comparative tests of various drug levels were conducted by using the same inoculating dose for one control and two treated groups. In one experiment, 24 chickens were used in each of three groups. One group served as controls, and the other two groups received 2 per cent and 1 per cent of the drug, respectively. In another experiment, three groups were employed in the same way in comparing the value of 0.5 per cent of the drug with the 2 per cent level. The combined results of these tests are presented in table 3.

In control groups, only 4 chickens (7.4%) among the total of 54 employed, proved resis-

*Coöperative tests with G. D. Quigley.

tant to inoculation, whereas in the 2 per cent, 1 per cent, and 0.5 per cent treated groups, respectively, 85.1 per cent, 86.6 per cent, and 80.6 per cent resisted experimental inoculation with cholera organisms. In control groups, 92.6 per cent sickened, and 85.1 per cent died. In the three treated groups considered in the previously mentioned order, death losses were 14.8 per cent, 10.0 per cent, and 16.4 per cent.

Blood Levels Obtained with Various Concentrations of Sulfathiazole in Feed†.—In studying the effect of various sulfathiazole levels on cholera, one or two blood samples were drawn from each fowl receiving medicated mash. These were tested for sulfathiazole, according to the method outlined by Bratton and Marshall.[6] Tests also were made in naturally infected flocks receiving treatment. Average concentrations of sulfathiazole in milligrams per 100 cc. of blood are presented in table 3.

TABLE 3—Blood Levels Obtained with Various Concentrations of Sulfathiazole in Feed

Sulfathiazole in feed (%)	Tested chickens (No.)	Tests (No.)	Ave. concentration sulfathiazole in blood: mg./100 c.c. blood
0.50	60	90	1.65
1.0	29	58	3.67
2.0	140	206	4.67

In 60 chickens receiving the drug at the 0.5 per cent level, a total of 90 tests was made. The average concentration of sulfathiazole was 1.65 mg./100 cc. of blood. In 29 chickens receiving 1 per cent of the drug in mash, 58 tests revealed an average concentration of 3.67 mg./100 cc. of blood. In 140 chickens receiving mash containing 2 per cent of the drug, a total of 206 tests was made. The average level of sulfathiazole in these groups in milligrams per 100 cc. of blood was 4.67.

In addition, sulfathiazole was administered at a 1 per cent level to several naturally infected turkey flocks. In one flock, 20 turkeys, selected at random after treatment, showed an average concentration of 2.45 mg./100 cc. of blood. In the other, an equal number of turkeys had an average concentration of 2.25 mg./100 cc. of blood. Although these levels are nearly equal, it was necessary to offer wet mash three times daily to the latter flock in order to stimulate feed consumption.

Sulfathiazole sodium administered intramuscularly in the quantity of 500 mg./6 lb. in nonlaying hens produced a blood concentration of 5 to 8 mg./100 cc. two hours after injection. Six hours after injection, the blood concentration had dropped to 1 mg./100 cc. of blood.

Effect of Sulfathiazole on the Minimum Lethal Dose of Pasteurella Avicida.—An effort was made to determine the trend of sulfathiazole effect on approximate minimum doses of *P. avicida* by administering graded doses of organisms to groups of treated and control chickens. The treated groups received mash containing 2 per cent of sulfathiazole. In preparing graded doses, a dense suspension first was made and from it 12 serial dilutions, so adjusted that each contained half the number of organisms in the preceding suspension. From most dense to dilute, these are designated in table 4 by letters (A) to (L) inclusive. The (A) suspension, therefore, contained 2,048 times the number of organisms in the (L) suspension. Eight chickens were injected intravenously with each suspension from (E) to (L), inclusive. Precautions to insure the injection of equal volumes of inoculums were taken. Half of the chickens injected with the various suspensions received mash containing the drug, and half were left as controls. In addition, 4 chickens receiving medicated mash were injected with each suspension from (A) to (D), inclusive. Since it was known that these inoculums exceeded the m. l. d. for the controls, parallel infections above the (E) dose were omitted.

It is convenient, but not statistically significant, to designate the livability in the various groups by percentage figures, according to whether 1, 2, 3, or 4 chickens survived the inoculation. A minimum lethal dose for control and treated groups is assigned to that suspension causing the death of all 4 chickens. Thus, among the controls, the suspension designated as (H) was credited with being the m. l. d., whereas in the treated group, the (A) suspension was the apparent m. l. d. The m. l. d. for the treated chickens, therefore, was 128 times greater than that of the controls. It is to be noted in the same

†Blood tests for sulfathiazole were conducted by Anita P. Holst.

table that certain control chickens in the (I) group withstood a dose of organisms eight times greater than the number causing the death of 2 other controls (L group). Thus, there is apparent within the control group a certain variation in resistance attributable to natural causes. Within the treated groups, a somewhat wider variation is indicated (B and F groups). The results of the experiment indicate, however, that the difference in m. l. d.'s, attributable to the treatment, was about twice as great as any ascribed to natural variation.

TABLE 4—Effect of Sulfathiazole Administered as 2 per cent of the Mash on the Minimum Lethal Dose of Pasteurella Avicida for Chickens

Inoculating dose		A	B	C	D	E	F	G	H	I	J	K	L
Progressive increase in numbers of organisms over L suspension		2048	1024	512	256	128	64	32	16	8	4	2	
Percentage of livability in the group	Controls							00	00	50	50	25	50
	Treated	00	25	75	25	75	75	100	100	100	100	100	100

Naturally Infected Flocks.—Sulfathiazole in concentrations of 0.5 per cent and 1 per cent of mash has been administered to a limited number of naturally infected flocks, in which it was felt that accurate records could be obtained. In three turkey flocks, the drug was administered at the level of 1 per cent to turkeys varying in age from 3 to 5 months. Losses stopped in three to four days, and the disease did not recur during the period before the turkeys were placed on the market. These flocks were all kept on wire platforms.

In two naturally infected chicken flocks, the drug was administered at the level of 0.5 per cent of the mash. In both flocks, the disease had existed in a chronic state for some time previously. Losses stopped in a few days after medication was started, and the flocks improved in appearance as well as in egg production. In each flock, however, the disease recurred in a few individuals after the drug was stopped.

Appearance of Cholera After Period of Medication.—In addition to recurring in naturally infected flocks, the disease appeared in a number of instances during the course of critical experiments after the period of medication. In the 22 chickens that became sick in treated groups, 10 were affected during the period of treatment and 12 after the administration of sulfathiazole had been discontinued. The average number of days from the end of the period of treatment to the appearance of symptoms was eighteen and one half days. The shortest and longest extent of the same period was two and forty-four days. *Pasteurella avicida* was recovered from the carcasses of all but 1 of these individuals.

TABLE 5—Appearance of Cholera After Period of Treatment

Total No. sick chickens in treated groups	Affected during period of treatment			Affected after period of treatment			Ave. No. days from end of treatment period to sickness
	Sick		Dead	Sick		Dead	
	(No.)	(%)	(No.)	(No.)	(%)	(No.)	
22	10	45.4	9	12	54.6	11	18.5

Discussion

Results obtained in these investigations indicate that sulfathiazole is effective to a considerable degree as an aid to the control of naturally induced cholera. It is unlikely that any blanket recommendation can be made regarding the amount of drug to be administered in all cases. Effective treatment will depend, as usual, on the skill and judgment of those prescribing for the affected flock.

Incorporation of such drugs for prophylactic purposes in feed as a selling point is not recommended. Reliance on the judgment of properly qualified veterinarians in administering treatment should greatly enhance beneficial results.

Preliminary experiments by the writer have indicated that the injection of killed suspensions of *P. avicida* (bacterins) creates an appreciable degree of immunity when measured two weeks after vaccination. It is entirely possible that the combined use of sulfonamides and bacterins may produce results somewhat superior to those herein reported.

Although a high percentage of treated chickens failed to develop symptoms after artificial

exposure to cholera, the average group body weight, as a rule, dropped slightly during the first week following inoculation. During the second week, however, the average body weight returned to normal in groups receiving sulfathiazole.

SUMMARY

1) Experimental fowl receiving 0.5 per cent to 2 per cent sulfathiazole in mash exhibited a significant degree of resistance against artificial exposure to fowl cholera.

2) Sulfathiazole administered at the 2 per cent level proved toxic for laying chickens in three to four days.

3) Average blood levels of 1.65 to 4.67 mg. /100 cc. of blood appeared effective against artificial exposure which produced high mortality rates in control groups.

4) The estimated group m. l. d. for small groups of chickens receiving sulfathiazole at the 2 per cent level was 128 times that of the controls.

5) Fowl cholera has occurred after the period of medication, both in experimental and naturally infected chicken flocks.

6) Resistance to fowl cholera is a variable factor. Some fowls survived a dose two to four times greater than that fatal to others.

References

[1]Cardoso, D. M., Nobrega, P., e Reis, J.: Acao Da Sulfanilamida Sobre A Infeccao Por "Pasteurella Avicida" Em Aves. Arquivos Do Instituto Biologico, Sao Paulo, Brasil, *9*, (Dec., 1938): article 23.

[2]Delaplane, J. P., and Stuart, H. O.: The Chemotherapeutic Value of Sulfathiazole in Preventing and Treating Infectious Coryza (*Hemophilus Gallinarum* Infection) in Chickens. J.A.V.M.A., *99*, (1941): 41-42.

[3]Severns, J. M., Roberts, E., and Card, L. E.: The Effect of Sulfonamids in Reducing Mortality from Pullorum Disease in Domestic Fowl. Poultry Sci., *24*, (1945): 155-158.

[4]Hammond, J. C.: Sulfonamids in the Control of Fowl Typhoid. Poultry Sci., *24*, (1945): 382-384.

[5]Kiser, J. S., Green, L. M., Prier, J., and Bottorff, C. A.: Treatment of Experimental and Naturally Occurring Fowl Cholera with Sulfamethazine. Poultry Sci., *26*, (1947): 546.

[6]Bratton, A. C., and Marshall, E. K., Jr.: New Coupling Component for Sulfanilamide Determination. J. Biol. Chem., *128*, (1939): 537-550.

DISCUSSION

DR. HURST.—You varied from 1.65 up to 4.67 mg./100 cc. of blood in sulfathiazole blood levels?

DR. DEVOLT.—That was in feeding sulfathiazole at different levels ranging from 0.5 to 2.0 per cent of the mash.

DR. HURST.—How did you feed it? Artificially?

DR. DEVOLT.—No indeed. We never had any trouble. I don't know whether I mentioned the fact, but in preliminary tests with a limited number of birds feed consumption was reduced about 13 per cent. At the 3 per cent level it was reduced much more than that. In one turkey flock we had trouble getting birds to eat this mash, but we never did have this difficulty in chicken flocks.

DR. DELAPLANE.—Did you feed 1 per cent or 2 per cent of sulfathiazole-treated mash?

DR. DEVOLT.—We fed 1 per cent.

DR. DURANT.—How long did you feed the medicated mash to the affected flock?

DR. DEVOLT.—Sixteen days, Dr. Durant.

DR. HURST.—At what time was the maximum blood level attained?

DR. DEVOLT.—That level would be attained within a few hours after the fowl started to eat the mash. We have injected some individual chickens intramuscularly with sulfathiazole sodium, 500 mg., and we found an average of 4 to 8 mg./100 cc. two hours after injection.

DR. MCDOUGLE.—How did you get the turkeys to eat the treated mash?

DR. DEVOLT.—One man fed a wet mash.

DR. MCDOUGLE.—Was milk used to wet the mash?

DR. DEVOLT.—No, it was just dampened with water, and they took it pretty well, the owner said.

DR. DELAPLANE.—Were any of these turkey flocks in egg production at the time you treated them?

DR. DEVOLT.—No, they were not. They were young birds ready for market.

DR. OSTEEN.—What method of exposure did you use?

DR. DEVOLT.—Intravenous inoculation of a culture for which the m.l.d. had been determined in previous tests.

DR. WERNICOFF.—Have you used the sulfathiazole in water to achieve the same results?

DR. DEVOLT.—No, we haven't tried that. Also, I don't doubt but that other sulfonamides may work as well. We continued with the one drug because we wanted the reference point.

Modified Newcastle Virus Vaccines

REGINALD L. REAGAN, MARY G. LILLIE, M.S.,
LEO J. POELMA, M.S., D.V.M., AND A. L. BRUECKNER, V.M.D.
College Park, Maryland

THE ADAPTATION of a California strain 11914 of Newcastle virus to the Syrian hamster[1] prompted experiments to test the value of this modified virus as an immunizing agent against Newcastle virus.

Komarov and Goldsmit[2] prepared modified virus vaccine* from brains of ducklings injected intracerebrally. After ten passages, the duckling virus injected into the wattle was not pathogenic for chickens. Birds so injected withstood challenge of Newcastle virus which killed 4 out of 5 control birds. Immunity seemed fully developed eighteen days after inoculation.

MATERIALS AND METHODS

Healthy, well-developed chickens from 4 to 10 weeks of age, from a Newcastle disease-free source, were used for our experiments, except in group 4, referred to later. No neutralizing antibodies for Newcastle virus have been found in any birds from the main source.

The following preliminary trial was conducted. One-half cubic centimeter of 10 per cent suspension of infected hamster brain of the seventeenth passage, which had an end point of 10^{-3} in hamsters, was injected into the base of the wattle of each of 4, 6-week-old chickens. Nine days after inoculation, 1 of the 4 chickens showed typical Newcastle disease symptoms. The remaining chickens were normal for thirty days after inoculation, at which time they were challenged by injection into the base of the wattle with virulent egg-propagated Newcastle virus, California strain 11914. This virus showed an end point of 10^{-8} in 10-day embryonated eggs and in 6-week-old chickens. Four normal chickens of the same age as those injected with the hamster-adapted virus were used as controls. The vaccinated group of 3 and the control group of 4 were challenged by injection of 0.2 cc. of 10^{-4} dilution of egg-propagated Newcastle virus. No symptoms of Newcastle disease appeared in the vaccinated birds during an observation period of a month following challenge, whereas the 4 chickens of the control group died of Newcastle disease on the fourth and sixth days.

Because the hamster-adapted Newcastle virus was sufficiently pathogenic after 17 passages in hamsters to produce Newcastle disease in 1 of 4 chickens, successive inoculations were made in hamsters through the twenty-ninth passage before further experimental trial. A pool of 10 per cent infected hamster brain suspension of the twenty-ninth passage, which showed an end point of 10^{-3} in hamsters and in embryonated eggs, was used in a second experiment. One-half cubic centimeter of this suspension was injected into the base of the wattle of each of 4 chickens. No symptoms of Newcastle disease appeared in any of these birds during an observation period of three weeks.

EXPERIMENTAL

One hundred and fifty healthy, 10-week-old, battery-raised chickens were divided into three groups of 50 each. One group remained under the regular battery conditions. The other two groups were moved into an unheated chicken house during winter weather. One week of exposure to cold was allowed for adaptation. The wattles of 42 birds were injected at the base with 0.5 cc. of 10 per cent suspension of infected hamster brain of the twenty-ninth passage, and 8 uninjected chickens were added as room contact controls to make group 1. The breast muscles of 42 birds were injected with 0.5 cc. of 10 per cent suspension of infected hamster virus of the twenty-ninth passage, and 8 uninjected chickens were included as room controls for group 2.

Twelve chickens succumbed after vaccination in groups 1 and 2, 6 from each unit. Four of the 12 died within the first forty-eight hours after injection. These were considered atypical deaths. The remaining 8 showed symptoms of Newcastle disease. Twenty-eight days after vaccination, all surviving chickens of groups 1 and 2 were challenged with 10^{-2}, 10^{-3}, and 10^{-4} dilutions of virulent egg-adapted Newcastle virus, California strain 11914.

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The authors acknowledge with appreciation the assistance of Dr. John Coffin of the staff.

*Reference should be made also to the egg-modified virus vaccines reported by Iyer and Dobson in 1940 since, according to latest reports, this product is far superior.—ED.

The remaining 50 chickens were used as controls for titration of the challenge virus. Results of the titration and immunization studies are presented in tables 1 and 2.

In table 1 the controls for groups 1 and 2, as well as the controls for groups 3, 4, and 5, were normal chickens which were challenged with 0.25 cc. of 10^{-2}, 10^{-3}, and 10^{-4} dilutions of the egg-adapted California strain 11914 of Newcastle virus. These controls compare directly with the vaccinated chickens of their corresponding groups.

Table 1 shows that the hamster-adapted virus protected slightly more than 80 per cent of the vaccinated chickens against an extremely large challenge dose. The two groups of 8 chickens which were used as room controls showed no symptoms of Newcastle disease while in contact with the vaccinated birds. Five of each group withstood 0.25 cc. of 10^{-3} dilution of virulent challenge virus.

The hamster-adapted virus of the twenty-ninth intracerebral passage produced typical Newcastle disease symptoms in approximately 10 per cent of the vaccinated birds. Hamster-adapted virus of the forty-ninth intracerebral passage, prepared from infected hamster brains ground with alundum and diluted with physiologic saline to a 10 per cent suspension, was used when an experiment was repeated with group 3. This suspension had an end point of 10^{-3} in young hamsters. Thirty chickens of this group were injected with 0.5 cc., 37 with 0.25 cc., and 37 with 0.1 cc. of a 10 per cent hamster brain suspension. Injection in each chicken in this group was made into the base of the wattle. Eleven unvaccinated chickens of group 3 were used as room contact controls.

Chickens of group 4 were injected with allantoic fluid collected from eggs that had been inoculated with hamster-adapted virus of the forty-ninth intracerebral passage. One-tenth cc. of the 10 per cent hamster brain suspension was inoculated into 11-day embryonated eggs which were incubated at 37 C. for three days. A pool of allantoic fluid was prepared from the embryonated eggs which appeared sluggish on the third day following

TABLE 1—Summary of Results Obtained with Several Sources of Living Modified Newcastle Virus

		Vaccination				Symptoms and deaths		Challenge		Results	
Group	No. of Birds	Vaccine	Amount in cc.	Site	Date	Typical	Atypical	Birds	Date	Died	Survived
1	42	hamster-adapted 29 sc	0.5	wattle	1/20/47	4	2	36	2/17/47	2	34
1 RC	8	....	...	...	....	0	0	8	2/17/47	3	5
2	42	hamster-adapted 29 sc	0.5	muscle	1/20/47	4	2	36	2/17/47	4	32
2 RC	8	....	...	...	....	0	0	8	2/17/47	3	5
1 and 2 controls	..	....	...	...	....	..	..	30	2/17/47	27	3
3	30	hamster-adapted 49 sc	0.5	wattle	3/21/47	1	10*	14[c]	4/18/47	0	14
	37		0.25	wattle	3/21/47	0	6*	26[c]	4/18/47	4	22
	37		0.1	wattle	3/21/47	1	10*	26	4/18/47	0	26
3 RC	11	....	...	...	....	0	3*	8	4/18/47	6	2
4	51	hamster-adapted allantoic fluid	0.5	wattle	3/21/47	17	2	32	4/18/47	3	29
	42		0.25	wattle	3/21/47	9	1	32	4/18/47	2	30
4 RC	7	....	...	...	....	0	2	5	4/18/47	3	2
5	40	hamster-adapted sheep brain	0.5	wattle	3/21/47	0	5**	30[c]	4/18/47	11	19
	50		0.25	wattle	3/21/47	0	4**	38[n]	4/18/47	13	25
5 RC	10	....	...	...	....	0	2**	8	4/18/47	6	2
3, 4, 5 controls	..	....	...	...	....	..	..	30	4/18/47	29*	1

RC = Room control unvaccinated contact chickens. sc = Subculture. [c] = Five used as contact controls. * = Coccidiosis, 18-24 days after vaccination. ** = Smothered by accident in chicken house. [n] = Eight used as contact controls.

injection. This fluid had an end point of 10^{-7} in 11-day embryonated eggs. A 10 per cent suspension of the allantoic fluid was used for vaccination of group 4.

In group 4, each of 51 chickens was injected into the base of the wattle with 0.5 cc. and each of 42 with 0.25 cc. of the 10 per cent allantoic fluid suspension. Seven unvaccinated chickens were used as room contact controls. Chickens of group 4 were purchased from a commercial source as "started chicks." Losses were extremely heavy in the lot prior to the start of the experiment. Whether the pathogenicity shown after challenge inoculation would be experienced with healthy chickens is problematic.

A large, nonpregnant ewe was injected intracerebrally with 5 cc. of 10 per cent hamster brain suspension of the thirty-sixth intracerebral passage. Five days after inoculation the sheep became sluggish and refused food. On the sixth day the sheep showed poor coördination while walking, rapid and labored respiration, and complete loss of appetite. On the seventh day the animal was unable to rise and, when lifted to its feet, would not move unless pushed vigorously. It remained standing for only a few minutes at a time. The vision seemed impaired. On the eighth day the animal was moribund; the respiration was labored and very rapid. Death occurred on this day, and in a few minutes the brain was removed. A 10 per cent suspension of the brain in physiologic saline solution was prepared in a Waring Blendor. This suspension, which had an end point of 10^{-2} in hamsters, was used for injection of chickens of group 5. Forty birds were injected with 0.5 cc. of 10 per cent sheep brain suspension and 50 chickens with 0.25 cc. of the same suspension. The base of the wattle was the site of inoculation. Ten untreated chickens were used as room contact controls.

TABLE 2—Immunization of Chickens Against Newasctle Disease with Modified Newcastle Virus*

Route of vaccination—group 1, base of wattle; group 2, breast muscle. Date of vaccination, 1/20/47.
Route of challenge—group 1 and 2, base of wattle. Date of challenge, 2/17/47.
Vaccine dose—0.5 cc. Challenge dose—0.25 cc. of various dilutions.

	Challenge Virus for Vaccinated Groups								
	Dilutions								
	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	LD_{50}**	Protection in LD_{50}
	S D	S D	S D	S D	S D	S D	S D		
Group 1	11 1	11 1	12 0					10^{-2}	446,700
Group 2	10 2	11 1	11 1					10^{-2}	446,700
	Challenge Virus Titration								
	1 9	1 9	1 9	0 5	0 5	0 5	2 3	$10^{-7.65}$	

*Infected hamster brain suspension prepared from 29th intracerebral passage used for immunization.
**The $L D_{50}$ was determined by the Reed-Muench technique.[3]
S = Survivors. D = Deaths.

All three vaccine preparations used for the experiments described were prepared and were stored in a dry ice (CO_2) box at a temperature of –40 to –60 F. until actual use.

Twenty-one days after inoculation several chickens of each of the groups were bled; the serums were pooled for the respective groups and tested for neutralizing antibodies, using 11-day embryonated eggs. There was a very low antibody titer on this trial but, when neutralization tests were repeated on similar groups of chickens twenty-seven days after inoculation, the antibody titer had increased.

All of the chickens injected with these various modified Newcastle virus suspensions were checked twice daily. In group 3, 2 chickens out of 105 showed symptoms of Newcastle disease; in group 4, 16 out of 93 showed symptoms; and in group 5, no symptoms of Newcastle disease were observed.

Twenty-eight days after vaccination 5 chickens of group 3 injected with 0.5 cc. and 5 chickens injected with 0.25 cc. hamster-adapted virus, 5 chickens of group 5 injected with 0.5 cc., and 8 chickens injected with 0.25 cc. of the sheep brain suspension were placed with 10^{-1} and 10^{-2} challenge virus

titration chickens. Twenty-two normal chickens of the same age were placed with the vaccinated chickens and the challenge virus titration birds.

None of the 23 vaccinated chickens showed symptoms of Newcastle disease during twenty-one days' exposure with the challenge virus titration chickens, whereas 9 of the 22 normal chickens showed typical Newcastle disease symptoms within the same observation period.

On the twenty-eighth day after injection, all vaccinated chickens except those removed for the contact experiment were challenged as shown in tables 1, 2, and 3. The challenge was egg-adapted virus of the eighteenth passage. The titration in normal chickens is shown in tables 2 and 3. This virulent Newcastle egg-adapted virus had an end point of 10^{-8} in 11-day embryonated eggs. The challenge dose was 0.25 cc. of the various dilutions injected into the base of the wattle. Those chickens used for the challenge virus titrations were the same age and development as those in the vaccinated groups.

Hamster-adapted Newcastle virus in the form of 10 per cent brain suspension in physiologic salt solution, as used for immunization studies, was tested for infectivity in hamsters after storage at 4 C. After two months' storage, the virus was still infective when injected intracerebrally into hamsters.

Hamster brain suspensions of passages 50 to 60 were injected into each of 10, 4-lb. rhesus monkeys by combined intramuscular, intradermal, and subcutaneous injections, covering a period of three weeks. These monkeys received a total of 15 cc. of 10 per cent infective hamster brain suspension. They showed no visible symptoms over a period of two months.

SUMMARY

Hamster-adapted Newcastle virus has shown decreasing pathogenicity for chickens with continued intracerebral hamster passage. The immunizing value of all hamster brain vaccines tested has been of a high order, both to challenge by injection and by contact.

Hamster-adapted Newcastle virus after one intracerebral passage in sheep proved nonpathogenic for chickens. The immunizing value of the sheep brain virus was of low order to challenge by injection but of high order to challenge by contact.

Hamster-adapted Newcastle virus after one chicken embryo passage proved pathogenic

TABLE 3—Immunization of Chickens Against Newcastle Disease with Modified Newcastle Virus

Route of vaccination: base of wattle. Date of vaccination: March 21, 1947.
Route of challenge: base of wattle. Date of challenge: April 18, 1947.
Dose of challenge: 0.25 cc. of various dilutions.

	Challenge virus for vaccinated groups											
	Dilutions											
Group	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	LD_{50}	Protection in LD_{50}
	S D	S D	S D	S D	S D	S D	S D	S D	S D	S D		
3*												
0.5 cc.		4 0	5 0	5 0							10^{-2}	120,200
0.25 cc.		6 3	6 1	10 0							10^{-2}	120,200
0.1 cc.		.. ..	13 0	13 0							10^{-3}	12,020
4**												
0.5 cc.		13 2	9 0	7 1							10^{-2}	120,200
0.25 cc.		8[n] 2	10 0	10 0							10^{-2}	120,200
5***												
0.5 cc.		5 5	9 1	5 5							$10^{-2.48}$	39,810
0.25 cc.		11 4	7 3	7 6							$10^{-2.2}$	75,860
	Challenge virus titration											
	1 10	1 9	0 10	0 10	1 9	5 5	7 7	6 4	7 3	10 0	$10^{-7.08}$	

*Hamster-adapted Newcastle virus in hamster brain suspension used as vaccine.
**Allantoic fluid infected with hamster-adapted Newcastle virus used as vaccine.
***Sheep brain suspension used as vaccine.
[n] = Two atypical deaths after challenge.
S = Survivors. D = Deaths.

for chickens. The immunizing value of the embryo vaccine was of high order to challenge by injection.

Susceptible birds, used as contact controls with birds injected with all vaccines, failed to show clinical evidence of spread of the vaccine strains; but there appeared to be some resistance to challenge injection in these birds, as compared to the regular controls.

Hamster-adapted Newcastle virus failed to produce abnormal symptoms when injected by several routes into rhesus monkeys.

Hamster-adapted Newcastle virus in brain suspension remained viable when stored at 4 C. for sixty days. It was pathogenic for hamsters when injected intracerebrally at the end of the storage period.

CONCLUSIONS

Hamster-adapted Newcastle virus of certain passages in the form of hamster brain suspension or embryo material gives promise of being valuable as an immunizing agent against Newcastle disease. The use of such virus as vaccine in the form of brain suspensions of other species which prove susceptible is also possible.

References

[1]Reagan, Reginald L., Lillie, Mary G., Poelma, Leo J., and Brueckner, A. L.: Transmission of the Virus of Newcastle Disease to the Syrian Hamster. Am. J. Vet. Res., *8*, (1947): 136-138.

[2]Komarov, A., and Goldsmit, L.: Preliminary Observation on the Modification of a Strain of Newcastle Disease Virus by Intracerebral Passage through Ducklings. The Vet. J., 102, (1946): 212-218.

[3]Reed, L. J., and Muench, H.: A Simple Method of Estimating the 50% End Points. Am. J. of Hyg. 27, (1938): 493-497.

DISCUSSION

DR. E. P. JOHNSON.—Dr. Delaplane, I would like to ask about the relative virulence of the California and the Eastern Newcastle disease strains in these experiments. Did the mortality run pretty high in the California strain? I understood it was much less virulent than the Eastern strain.

CHAIRMAN POMEROY.—Whom would you like to answer that question?

DR. JOHNSON.—I think Dr. Brueckner.

DR. BRUECKNER.—We have done no work, Dr. Johnson, with any other than this one California strain. In our first two groups, the titration showed that the California virus that we were using there was 10 minus 7.65, and the other experiment ran 10 minus 7.08. We have worked with no strain of virus other than the California strain, so I couldn't attempt to compare them.

In 1 case, with the same dilution which we used to challenge our vaccinated birds, we killed 27 out of 30 and in the other case, 29 out of 30. I have done no work on comparative pathogenicity of the strains, so I wouldn't be able to give an answer.

DR. C. A. BRANDLY.—The California strain reported on here by Dr. Brueckner was apparently the one isolated in eggs by my colleagues and me at Harvard, in 1944, from infected living material supplied by Dr. J. R. Beach of California and designated, according to his accession number, as 11914. Various differences have been demonstrated among strains of Newcastle virus, as has been the case with many other kinds of viruses. I think it would be very unfortunate if some effort is not to be made now and in the future to designate the particular strain of Newcastle virus used in any experiments as to source, number, and other pertinent information.

If someone would take the Rawlings strain of typhoid and designate it by some other term, much needless confusion would result. I would suggest we all make a serious effort, wherever possible, to define each strain of Newcastle virus used in our work. Perhaps Dr. Schoening can clarify the point as to what the Bureau sends out as strain 15, whether it is a new strain or whether, as some of us have the impression, it is merely 11914, California.

DR. OSTEEN.—It is 11914. Fifteen designated the embryo passage since it was received by the Bureau.

DR. BRANDLY.—But it doesn't indicate source or identity.

DR. OSTEEN.—That is all—just "fifteenth passage."

DR. SCHOENING.—We will take cognizance of that, and those to whom we have sent this virus, if it hasn't been definitely identified by the California number, we will see that it is done. I think it is quite important that the identity of that particular strain be kept.

DR. OSTEEN.—I think that the responsibility of the error today is mine, but I do know that the California strain of virus that has been sent out by our laboratory as 15 is 11914.

DR. BRUECKNER.—We didn't give it a number. We described it in the first paper on the adaptation to the hamster as the strain that Brandly recovered in Boston from material from Dr. Beach, and which we received from the Bureau. Since we now have the number of it, we can carry it as such. We can identify it as being that particular strain.

CHAIRMAN POMEROY.—I think that this is a very important point for all of us who are using the virus. We also happen to be using this strain of the virus which we got from the Bureau.

Electric Ejaculation

Determination of Optimum Electric Shock to Produce Ejaculation in Chinchillas and Guinea Pigs

CHARLES F. DALZIEL, B.S., M.S., E.E., and CHESTER L. PHILLIPS, B.S.
Berkeley, California

THIS PAPER presents improvements in the technique of electric ejaculation with emphasis given to safety and effectiveness. Concern has been given to the safety of both the operator and animal and secondary concern to effectiveness and discomfort of the animal. The experiments have been conducted in the electrical engineering laboratories at the University of California, Berkeley.

Although artificial ejaculation and insemination are currently practiced in breeding cattle and sheep, techniques applicable to smaller animals have not yet been sufficiently perfected to invite widespread use. It is difficult to employ methods applicable to larger animals, such as massage, vaginal sheaths, artificial vaginas, to smaller animals due to the small size of penis. Although selective breeding is widely used in improving certain strains, the method is of no avail for monogamous animals. Selective breeding may have other disadvantages, as certain animals sometimes fight their mates at breeding season. The improved techniques of electrical ejaculation presented herein are offered as a scientific and reasonably safe approach to the problem. In addition, the findings may be of value to biologists engaged in pure research.

Preliminary tests on guinea pigs, using commercial 60-cy. (cycle) a.c. (alternating current) limited to 8 ma. (milliamperes), produced such pain and violent reactions that immediate experimentation with the more costly animals was considered inadvisable. As a result of these early experiments, a painstaking investigation using guinea pigs was made to determine satisfactory techniques before proceeding with chinchillas. The experiments were conducted at weekly intervals throughout the academic year 1941-1942, and except for one or two instances, the animals were subjected to only one test per week. No permanent injuries were observed, and subsequent natural breeding confirmed the opinion that the method, admittedly severe, produced no harm to the animals. Since the object of this paper concerns improvements in the method of electric ejaculation, the biologic aspects of the problem will not be discussed. However, it may be of interest to mention that 9 Bolivian-Imperial chinchillas, believed to be the only half-breeds in existence, were produced as a result of the investigation.

HISTORICAL REVIEW

Although the exact mechanism by which an electric shock produces ejaculation is not known, erection of the penis and ejaculation have been observed in individuals electrocuted, and in those hanged. Batelli[1] was the first to describe an experimental procedure in which an electrical stimulation applied to the base of the brain of a mature male guinea pig produced ejaculation. Battelli applied shock stimuli of about 30 V (volts), 47-cy. a.c., with a shock duration of about two seconds, and a period between shocks of one to two minutes.

Moore and Gallagher[2] confirmed the findings of Battelli, and report more than 2,000 electric shocks given to over 100 different animals with only two deaths attributed to electric shock. In their experiments, male guinea pigs were lightly anesthetized and given successive electric shocks of approximately 33 V a.c. (frequently not specified) applied on the head. One electrode, an ordinary needle, was pushed through the skin on the back of the neck so that the point came to rest at the base of the skull; the second electrode, a blunt, rounded metal terminal, was inserted into the guinea pig's mouth. Application of the current send-

Associate professor (Dalziel), division of electrical engineering, and senior student (Phillips) in electrical engineering, University of California, Berkeley, at time study was made, now in the industrial division, General Electric Company, San Francisco, Calif.

Acknowledgment is given James F. Mitchell, practicing veterinarian and chinchilla rancher, for suggesting the problem and for supplying and handling the animals. Acknowledgment is also due Edward J. Crawford, chinchilla rancher, for his assistance in supplying and handling the animals.

ing a shock through the brain caused a vigorous general body contraction, with the result that a typical ejaculation was produced. As a routine, three successive shocks of three seconds duration were applied at one-minute intervals. The first stimulation usually caused little or no secretion; the second stimulation was accompanied by the discharge of the majority of the contents of the accessory reproductive organs; and a third stimulation resulted in almost complete elimination of their contents.

In 1936, Gunn[3] published a comprehensive report covering over 12,000 electric shocks given to more than 100 Australian rams on 600 different occasions. In Gunn's method, one electrode, a stout needle, was inserted into the longissimus dorsi muscle, which is located between the fourth lumbar vertebra and the last sacral vertebra. The second, a smooth rod about the diameter of an ordinary lead pencil, was inserted about four inches into the previously emptied rectum. He recommends using about 30 V (resulting current 160-190 ma), 50-cy., a.c., although direct current interrupted and reversed to correspond to 50 cy. was also used with satisfactory results. The recommended stimuli consisted of ten to 20 electric shocks, of five to ten seconds duration or less, with corresponding off periods between shocks. The semen was normal in all respects, but in general the first portion of the ejaculate was thin and watery in appearance. The main bulk of the ejaculate, containing most of the sperm, was obtained from the second or third to twelfth or thirteenth stimuli. Considerable variation was observed between different rams in the number of stimuli required to produce a good ejaculation. All attempts to maintain general anesthesia in rams during electric shock stimulations were unsuccessful, as rams in deep third stage anesthesia, on the first application of the stimulus, rapidly revived. They could only with difficulty be anesthetized again.

Gunn conducted preliminary experiments in which successful electric ejaculations were obtained on several other species of animals, including guinea pigs, dogs, and goats. Experiments using guinea pigs were made to compare the efficiency of ejaculation produced with electrodes on the head and electrodes placed in the anus and lumbar regions. No significant differences in the ejaculate were found. However, 3 guinea pigs died as a result of applying the shocks to the head, and contacts on the head were abandoned in favor of the rectum-lumbar regions to reduce the electric shock hazard. The experiments on rams were conducted with only one electrocution. In this case, the electric shock was administered without previously having measured either the current or the voltage.

Thomas Durfee, *et al.*,[4] conducted experiments on albino rats and rabbits to determine the best electrode positions. The electrode positions used on the rats included mouth to nape of neck, mouth to ear, mouth to lower jaw, and rectum (electrode inserted about 1½ in.) to the third sacral vertebra. Three deaths were reported due to electric shock, two of which occurred when the shocks were applied on the head. Electrode positions used on the rabbits included mouth to nape of neck, and rectum (electrode inserted about 3 in.) to the first or second sacral vertebra. Best shock conditions of both animals were obtained with electrodes in the rectum-sacral regions, and were 13 V, 15 ma., with the shock duration two to four seconds, and an interval between shocks of thirty to sixty seconds. Sixty-cycle a. c. was used in these experiments.

Benham and Enders[5] developed an electronic device for producing electric ejaculation in small animals, and applied it to rats and foxes. In use on foxes, one electrode was placed in the rectum about 3 in. past the anal sphincter; the second electrode, a needle, was inserted in the muscles of the spine near the third lumbar vertebra. The device could be set for frequencies between 20 to 60 cy., and the wave form of the output could be controlled from a sharp positive peak, with no corresponding negative peak, to the full sine wave similar to that obtained from commercial power sources. The stimuli given the foxes consisted of two five-second shocks, with an interval between shocks of about thirty seconds, frequency 25-cy., 40 V, and 30 ma. These investigators venture the opinion that a wave form consisting of successive positive peaks produced the most efficient stimulus, together with minimum discomfort and hazard to the animal.

Unfortunately, insufficient data are included in their paper to substantiate this conclusion. Since no mention was made of whether the voltage and current readings were rms. or crest values, the apparent reduction in reaction time on sharply peaked waves is open to question. Due to biologic variability between different animals, and in the same animal at different times, this type of response must be analyzed using statistical methods. Valid conclusions are obtained only when they are derived from a considerable number of tests. Although the number of tests conducted was not mentioned, it is stated that only a relatively small number were made. Thus, the apparent reduction in shock required to produce ejaculation may not be representative of that to be expected from a large sample. However, the suggestion regarding the possible advantages of peaked wave forms deserves serious consideration.

Analysis and Results

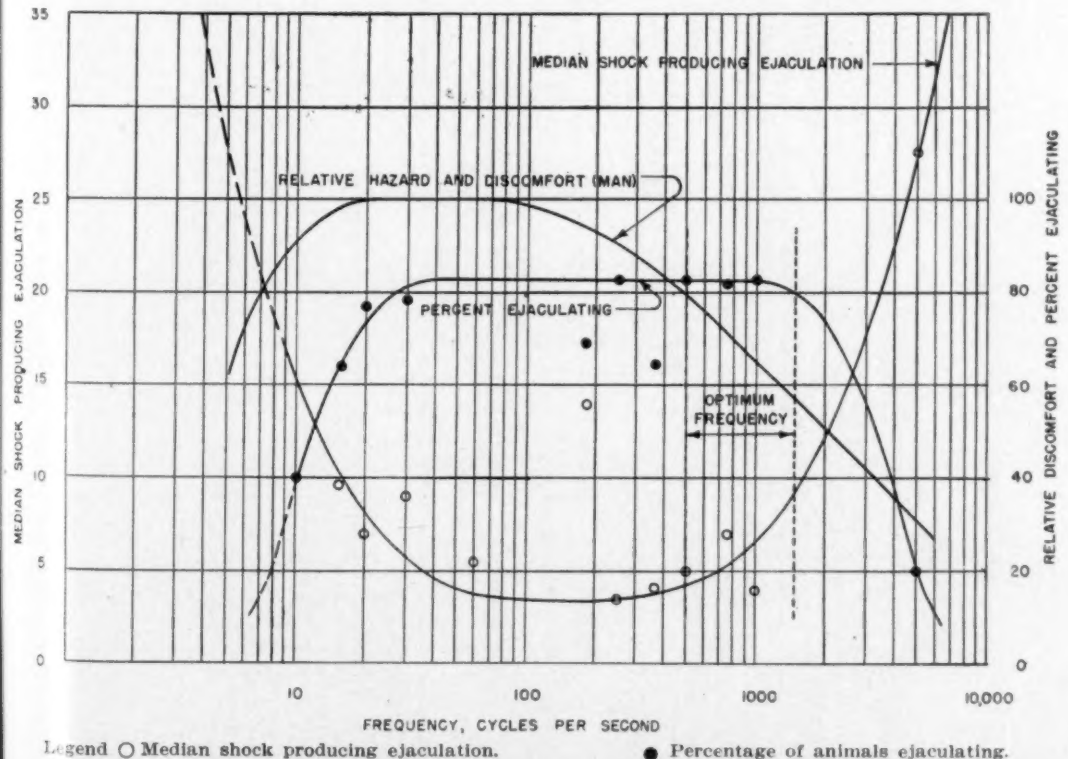
MacMahon[6] states that during very severe electric shocks, that is, shocks just insufficient to cause death, the animal is rigid, the heart stops beating, and respiration ceases. Shocks of this severity may also cause eversion of the anus, defecation, micturition, vomiting, and emission. After shock, the animal shows general weakness, paralysis of posterior extremities, and diminished sensations. Due to the relatively small currents used in the present investigation, the effects of shock were much less pronounced, and, although the animals manifested considerable discomfort, all symptoms subsided immediately upon interruption of the current. No ill effects on general health or fertility in the animals were observed.

The six factors controlling electric shock intensity and hazard listed in approximate order of importance are:

1) Current pathway through the body.
2) Magnitude of the current.
3) Shock duration.
4) Frequency.
5) Wave shape.
6) Phase of the heart cycle at the instant the shock is applied.

The most dangerous current pathway is one involving the heart. Ferris, *et al.*,[7] has shown that the least dangerous current pathway involving the trunk is from one hind leg to the other. However, preliminary experiments using this pathway on guinea pigs produced no stimulation of the reproductive organs. Similarly, unsuccessful results were obtained with electrodes in the anus-stomach regions. Copious ejaculations were obtained with electrodes placed in the anus-lumbar regions, and, since this current pathway is short, the current reaching the heart should be small. This current pathway is believed to be the safest, and it was used in all subsequent experiments.

During the subject series of experiments the electrode for insertion into the anus consisted of a polished rounded brass rod ⅛ in. diameter and 1⅝ in. long. Connection to the electrode was made by a smooth rubber insulated, flexible copper wire of slightly smaller outside diameter, which was soldered in a small hole drilled in one end of the rod. Before insertion, the rod was wetted with saline solution to decrease friction, to insure good contact, and to reduce the possibility of burns. The other electrode consisted of a small copper washer (size of a penny) with two holes drilled dia-



Legend ○ Median shock producing ejaculation. ● Percentage of animals ejaculating.

Chart 1—Effect of frequency on electric ejaculation combined results of experiments on guinea pigs and chinchillas. Shock intensity: 8 ma.; duration 3 seconds on period, 12 seconds between successive shocks.

TABLE I—Effect of Frequency on Electric Ejaculation

Combined Results for Guinea Pigs and Chinchillas.

Frequency	10	15	20	30	60	180	250	360	500	750	1000	5000	10000
Number of shocks required to produce ejaculation	4	2	*10* 1	2	2	2	*1*	1	1	2	1	27	34n
	6	3	*12* 2	4	3	5	*1*	2	1	4	1	29	*3½n*
	34n	5	13 3	*6*	3	6	2	2	3	6	2	*3½n*	34n
	34n	8	15 3	9	3	8	3	4	4	6	2	34n	*3½n*
	34n	9	19 4	9	5	14	3	8	4	7	4	*3½n*	34n
		10	21 4	19	6	15	¼	12	5	9	¼	34n	*3½n*
		10	*21* 6	23	7	15	7	*13*	5	*19*	4	*3½n*	34n
		11	25 *6*	*33n*	5n	17	9	*3½n*	*6*	*23*	5	34n	*3½n*
		11	27 6	*3½n*	5n	*33*	*17*	*3½n*	9	24	7	*3½n*	34n
		17	33n 7		15n	*3½n*	17	*3½n*	11	*3½n*	13	34n	*3½n*
		34n	33n 7			34n	34n	34n	*23*	34n	16		34n
		34n	33n 7			*3½n*	34n		*3½n*		*3½n*		
		34n	33n 7			*3½n*							
		34n	*3½n* 8										
		3½n	*3½n* 9										
		34n	*3½n* 9										
			3½n										
Number of Tests	5	16	33	9	10*	13	12	11	12	11	12	10	11
Percentage ejaculating	40	63	76	78	..	69	83	64	83	82	83	20	0
Medium shock producing ejaculation		9.5	7	9	5.5	14	3.5	4	5	7	4	28	34n
Maximum V across animal	57	75	52	87	42	29	59	38	26	24	26	51	48
Median V across animal	29	31	22	42.5	37	22	23.5	20	20	20.5	21	14.5	11
Minimum V across animal	21	18	15	35	30	16	17	17	10	15	14	10	6

Shock current, 8 ma.; duration: 3 seconds on, 12-second rest period between shocks.

*Preliminary tests, shock duration: 5 seconds on, 10-second rest period.

Italics denote values for chinchillas; others for guinea pigs.
Letter "n" following number indicates no ejaculate at end of this number of successive shocks.

TABLE 2—Effect of Current on Electric Ejaculation

	Guinea Pig Number											Chinchilla Number								
	1	2	3	4	5	6	7	8	9	10	13	6886	6885	3497	3737	829	1115	4681	15	6779
							Current 6 ma									Frequency 30 cy.				
Voltage							37	27	35	31										
Shocks to produce ejaculation							29	33n	34n	33n										
							Median shock producing ejaculation—29.													
							Current 8 ma									Frequency 30 cy.				
Voltage	43	43	35	..	42							37			43		42	87		
Shocks to produce ejaculation	19	4	2	23	33n							9			34n		6	9		
							Median shock producing ejaculation—9.													
							Current 8 ma									Frequency 360 cy.				
Voltage		19		26	20	25		20			17	30	20	19	19	38				
Shocks to produce ejaculation		4		12	8	34n		2			1	13	2	34n	34n	34n				
							Median shock producing ejaculation—4.													
							Current 16 ma									Frequency 360 cy.				
Voltage	35	44				26	20	20											44	44
Shocks to produce ejaculation	5	34n				19	4	2											1	2
							Median shock producing ejaculation—3.													
Concluding test 3-second shock, current-ma						150*	220*	220*												
							Current 24 ma									Frequency 360 cy.				
Voltage	21	20	20	20	20							37	37	37	27					
Shocks to produce ejaculation	4	2	2	2	7							3	3	4	17n					
							Median shock producing ejaculation—3.													
Concluding test 3-second shock, current-ma	72	72	100	100	150*															

Shock duration: 3 seconds on; 12-second rest period between shocks.

Letter "n" following number indicates no ejaculation produced at end of this number of successive shocks.

*No ejaculate.

metrically opposite near the edges. The area around the third lumbar was thoroughly wetted with saline solution, a string was passed through the holes and around the animal to assist holding the electrode in place. The animal was then placed on its back, with the attendant's hand holding the electrode in position; the other hand (in a leather glove) was used to restrain the animal and keep it from biting. A second attendant inserted the anus electrode 1 to 2 in. in the rectum, and stood ready to collect the semen in a sterile test tube. A third attendant controlled the electrical apparatus and administered the shocks. Proper location of the electrodes in animals of different species is apparently critical, and it is probably best found by trial and error. An electrode held in position on the back is preferred to the needle electrode used by previous investigators.

Although the places of application and the electrodes were thoroughly wetted with saline solution, all attempts to obtain uniform contact resistances were unsuccessful. For example, in one test the extreme values of body and contact resistances of 11 animals were 6,000 and 750 ohms. As a result, the voltages required to maintain a preselected current through the animals varied considerably. Some variation in resistance was encountered during each test, however, usually the change in resistance was so small that the apparatus could be readjusted during a three-second shock period to maintain the current constant within $\pm$ 0.2 ma. The maximum, median, and minimum rms. (root-mean-square) voltages required to pass a current of 8 rms. ma. through the guinea pigs and chinchillas are given in table 1. Based on the above, it was concluded that the wide range of voltages precludes the use of voltage measurements for controlling or indicating the intensity of the electric shock. This conclusion is in complete agreement with the generally accepted (but often overlooked) fact that current and *not* voltage is the proper criterion for specifying the intensity of an electric shock.

A large number of tests were made to determine the current required to produce ejaculation. Significant results are given in table 2, from which it is noted that about 8 rms. ma. is close to a practical minimum. This value was used in the majority of the tests in order to evaluate other factors controlling optimum shock conditions. Sixteen milliamperes produced more effective results, and it is suggested that future work on small animals be conducted using shock currents up to this magnitude. The quantity of semen increased, as did the animal's discomfort, up to about 100 ma. Currents in excess of 100 ma produced violent reactions, so violent, in fact, that the life of the animal was endangered. The muscular contractions caused by shocks of this magnitude were so severe that no external secretion was produced. The maximum current given the guinea pigs was 220 ma; the maximum given the chinchillas was 24 ma. Most of the animals subjected to currents of 24 ma or more showed immobility of the hind legs. However, this condition rapidly passed off, and no ill after effects have been attributed to electric shock during the five-year interval from completion of the experiments to the date of writing (October 1947). It was observed that the first shock of a series produced the greatest discomfort. As a means of circumventing the severity of the first shock, future apparatus might be provided with a means to limit the current to one half or two thirds of the current used for succeeding shocks.

Ejaculation generally occurred just after interruption of the current, and although continuous shocks up to about thirty seconds were used, more satisfactory results were obtained by applying a series of short shocks. A rotary cam-operated, double pole switch was provided; one pole interrupted the shock current and the second energized a synchronous timer which gave an accurate record of shock duration. Although satisfactory preliminary tests were conducted using a shock duration of five seconds, and a rest period between shocks of ten seconds, decrease in discomfort without apparent decrease in efficiency resulted from shock durations of three seconds, and an off period of twelve seconds. This was used in all but the rather limited tests made on 60-cy., as indicated in the footnote of table 1.

The most important finding of the present investigation concerns the effects of frequency. Pertinent data are given in table 1 and are represented graphically in chart 1. Experiments were made to investigate the frequency response over a range from 0.8 to 10,000 cy. For frequencies of 360, and 60 cy. or below, synchronous generators driven by d.c. motors were employed in which both armature and field voltage control were used. For the other frequencies, a vacuum tube oscillator and amplifier were used. A cathode-ray oscilloscope was connected to monitor the output, and the wave form was maintained sinusoidal. Although one ejaculation was obtained using a frequency of 0.8 cy., 8 ma., and a continuous shock of nine seconds, the response on frequencies below 10 cy. was erratic and caused great discomfort to the animals. Frequencies of 10 to 20 cy. produced less discomfort, and a considerable amount of data was taken at 15 and 20 cy. before proceeding to higher frequencies. For frequencies of 20 cy. and below, the muscular contractions followed the current pulsations and the animal emitted piercing shrieks, in synchronism with the peaks of the current wave. For frequencies in the neigh-

borhood of 60 cy., muscular contractions were severe, and the animals exhibited signs of considerable distress. For frequencies above 120 cy., the muscles were unable to follow the rapid current pulsations, and the animal emitted intermittent shrieks at an almost constant rate of one to three per second. Both muscular contractions and the intensity of the shrieks decreased as the frequency was increased, indicating reduction in discomfort. It was, of course, very difficult to evaluate the distress of the animal. The relative hazard and discomfort curve of chart 1 was obtained by the senior author during exhaustive research to determine the effects of electric shock on man.[8, 9] In so far as could be determined, the curve also applies to these animals for frequencies above about 10 cy.

Due to the variation in the number of shocks required to produce ejaculation in different animals, and in the same animal at different times, and to the limited number of animals available, it was difficult to evaluate the response for guinea pigs and chinchillas separately. For a given frequency, the variations in the number of shocks within a group of the same species were generally larger than corresponding differences between the two groups of animals. For this reason, the data for both guinea pigs and chinchillas were combined and treated as a homogeneous sample to obtain a sufficient number of values on which to base conclusions. Table 1 shows the trend of the response with frequency in which the number of shocks required to produce ejaculation are tabulated in descending order. Data for chinchillas are shown in italics; the other values are for guinea pigs. The authors are of the opinion that a sufficient number of values were obtained to permit a fairly accurate determination of the frequency response. The median shock producing ejaculation and the percentage ejaculating during the first 34 successive shocks are believed the most important measures of response. In chart 1, smooth curves governed by the majority of the experimental points were drawn to show the median shock producing ejaculation and the per cent ejaculating as a function of frequency. In this series of tests the animals were given successive 8 rms. ma., shocks consisting of an on-period of three seconds and a twelve-second interval between shocks, until a total of 33 or 34 shocks had been applied. Animals not ejaculating by the completion of this number of shocks were rejected on the assumption that ejaculation was not forthcoming due to physiologic factors beyond the control of the investigators. Since the tests were stopped after the thirty-third or thirty-fourth shock regardless of whether or not ejaculation occurred, the median shock producing ejaculation (not the average) was used as a measure of the response.* Perhaps the best justification for combining the data was that for the majority of the tests, the median shock producing ejaculation for either guinea pigs or chinchillas was substantially the same as that obtained when the data were combined. Considering the limited number of tests on any one frequency, the consistency of the data are gratifying. The response was erratic, and insufficient data were obtained to determine the curves for frequencies of 10 cy. and below. However, it is believed that the curves diverge as indicated by the dash lines below about 9 cy., and again for frequencies above 2,000 cy. With exception of frequencies in the neighborhood of 180 cy., the response for intermediate frequencies was practically constant. Unfortunately, insufficient information was obtained to explain the poor response obtained at 180 cy. All factors considered, frequencies between 500 and 1,500 cy. are believed to be the optimum for producing ejaculation. Within this range, the median shock producing ejaculation is low, the per cent of animals ejaculating is high, the animal distress is reduced to a satisfactory level, and the voltage requirements have almost reached a minimum.

Sine wave shapes as determined from visual inspection of a cathode-ray oscilloscope were maintained in these tests. Circumstances due to the war shortened these investigations, thereby precluding tests to determine effects of wave shape. Reduction of electric shock hazard and discomfort, and possible increases in effectiveness are apparently of sufficient moment to warrant thorough investigation.

Ferris, *et al.*,[7] has shown that for short shocks, that is, for shocks of the duration of one heartbeat or less, the instant the shock is applied with reference to the phase of the heart cycle is extremely important from the standpoint of producing ventricullar fibrillation (instantaneous electrocution). Since the three-second shocks used in these experiments greatly exceed the duration of one heartbeat, it is believed that this factor is of minor importance in the present instance.

*The median is the value corresponding to percentile 50. The median rather than the average was used in this investigation because of the limited amount of data obtained on any one frequency. For example, consider frequency 250. The number of tests is 12, the number of shocks producing ejaculation is 10. The minimum number of shocks producing ejaculation is 1 and the maximum is 17. The median shock producing ejaculation is the middle value between 1 and 17 and is 3.5 (interpolated). The average shock producing ejaculation is the sum of the values above the two 34n's divided by 10 and is 6.4. In this case, the two values of 17 cause the average to be higher than might have been the case had a much larger number of tests been made, since the average approaches the median as a large number of points approach normal distribution. Obviously the per cent ejaculating is $10/12 = 83$.